

ARGININE VASOPRESSIN IN FOETAL SKELETAL MUSCLE

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I declare that this thesis is of my own composition, and that the work described within is my own, and was carried out by me.



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To Dave Carr, and everyone who misses him

Death has no power to end a friendship. It can only interrupt your friendship, for love is a force more powerful than death. And friendship is surely a lasting testament to love's power.

...for you yourself are the most lasting memorial to your friend. That person's thumbprints are visible in your clay. You are living testimony that your friend's life made a lasting difference.

- Carol Luebering

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ABSTRACT

Arginine Vasopressin (AVP) is also known as the anti-diuretic hormone (ADH). The two regulatory effects that provided these names (increasing blood pressure by causing vasoconstriction, and reducing water loss by promoting water re-absorption in the kidney) are commonly described as the only physiologically relevant functions of this peptide. However, other cellular effects of AVP have also been described both *in vitro* and *in vivo*. For example, in the adult mammal, AVP has been shown to play a role in platelet aggregation, hepatic glycogenolysis, and memory consolidation.

Studies described in this thesis set out to examine a potential additional function for AVP. Data were published suggesting there is a significant amount of AVP immunoreactivity (AVP-ir) in foetal human skeletal muscle. AVP-ir was described at concentrations significantly higher than circulating levels of AVP, and was shown to decrease with increased gestation age. Also, in rat myogenic cell lines, incubation with AVP resulted in promotion of fusion and up-regulation of muscle specific gene expression. This effect was described as being mediated by the V1a vasopressin receptor. Taken together, these results suggest a role for vasopressin in skeletal muscle development and point to an additional alternative site for the synthesis of biologically active AVP. An extraction method was developed using solid phase extraction (SPE) followed by radioimmunoassay. The physical recovery of the SPE step was reproducibly better than 70% when extracting AVP from homogenised muscle tissues. The radioimmunoassay had a cross reactivity of less than 0.01% with both oxytocin and arginine vasotocin. This extraction method was developed in response to the demonstration that the direct assay of acidified extracts could not provide an accurate measurement of AVP in extracted muscle. It was demonstrated that acid present in the samples will interfere with the assay, and it is argued that this artefact probably gave rise to the AVP-ir reported previously.

AVP-ir in foetal muscle samples using SPE was not significant. This was in contrast to levels found in positive control tissues - foetal human adrenal and pituitary glands and adult rat adrenal glands - where AVP-ir levels were in agreement with previously published data. A relationship between the age of the foetus and the vasopressin contained in the adrenal gland was observed. Rat adrenal gland contained significant amounts of AVP-ir, and when immunohistochemistry was used to confirm these findings, regional distribution of AVP-ir in the rat adrenal medulla tissue was observed. In Western blot analysis of extract of foetal muscle, bands were seen at appropriate molecular weights using a specific antibody to the V1a receptor.

Concentrations of AVP required *in vitro* to cause fusion of muscle cells and concomitant expression of relevant muscle specific genes could not be demonstrated in any sampled human foetal muscle. Evidence exists that there is a role for AVP in the regulation of carbohydrate metabolism in skeletal muscle, thus explaining the presence of the receptors without the need for participation in the process of muscle development. On this basis, it is concluded that it is unlikely that vasopressin is involved in the development of skeletal muscle in the human foetus. The discovery of a regional distribution of AVP-ir in the rat adrenal gland merits further investigation of the role of extra hypothalamic vasopressin in this organ.

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LIST OF ABBREVIATIONS USED

ACN	Acetonitrile
ACh	Acetylcholine
ACTH	Adrenocorticotrophic hormone
APUD	Amine precursor uptake and decarboxylation
ADH	Anti-diuretic hormone
Arg	Arginine
AVP	Arginine-8-vasopressin - also referred to as vasopressin
AVP-ir	Arginine-8-vasopressin immunoreactivity
AVT	Arginine vasotocin
ABC	Avidin-biotin enzyme complex
BSA	Bovine serum albumin
cAMP	Cyclic AMP
C8 silica	Octylsilyl-silica
cDNA	Complimentary deoxyribonucleic acid
CRH	Corticotrophin-releasing hormone
Da	Dalton
DHMC	Dartmouth-Hitchcock Medical Centre
DAG	Diacylglycerol
DAB	Diaminobenzidine
DTT	Dithiothreitol
DAGs	Dystrophin associated glycoproteins
FMAF	Faculty of Medicine Animal Facility
g	Gram
<i>gonzo</i>	anti-vasopressin rabbit polyclonal antiserum
GPCRs	G - protein coupled receptors
³ H-AVP	AVP labelled with tritium
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
HCl	Hydrochloric acid
¹²⁵ I-AVP	AVP labelled with iodine-125
IHC	Immunohistochemistry
Lys	Lysine
LVP	Lysine-[8] vasopressin
mRNA	Messenger ribonucleic acid
μl	Microlitre (1x10 ⁻⁶)
ml	Millilitre (1x10 ⁻³)
M	Molar (moles/litre)
MWCO	Molecular weight cut off
ng	Nanogram (1x10 ⁻⁹)
NHP	Neurohypophyseal
OT	Oxytocin
PVN	Paraventricular nucleus
PAM	Peptidyl-glycine alpha-amidating monooxygenase
PMSF	Phenylmethylsulfonylfluoride
PBS	Phosphate buffered saline
pg	Picogram (10 ⁻¹²)

PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
RIA	Radioimmunoassay
RT-PCR	Reverse transcriptase-polymerase chain reaction
<i>s278</i>	anti-AVP sheep polyclonal antibody
SAPU	Scottish Antibody Production Unit
SDS	Sodium dodecylsulphate
SCN	Suprachiasmatic nucleus
SON	Supraoptic nucleus
TFA	Trifluoroacetic acid
<i>tg1</i>	anti-AVP rabbit polyclonal antiserum
<i>vivian</i>	anti-V1a-vasopressin receptor rabbit polyclonal antiserum
VAG	Vasopressin-associated glycopeptide
VLP	Vasopressin-like peptide
v/v	Volume/volume
w/v	Weight/volume

Chapter 1

Introduction

1.1 Overview

Arginine-8-vasopressin (AVP - also referred to as vasopressin) is also known as the anti-diuretic hormone (ADH). Study of this peptide has proceeded for almost a hundred years largely on the basis that vasopressin has an exclusive source and a defined set of targets. That is, it is synthesised in the cell bodies of magnocellular neurones grouped together in a number of tracts of the anterior hypothalamus, transported along their axons to be stored in, and secreted from, the nerve endings that reside in the posterior pituitary gland. Once secreted into the blood stream, the peptide hormone circulates either to the kidneys where it exerts an anti-diuretic effect, or acts on smooth muscle cells in blood vessels, resulting in a vasopressor effect. These actions of vasopressin were first described and demonstrated in remarkable experiments either using whole organ preparations or whole animal studies and the two names subsequently arose from the two roles designated as the primary functions of this peptide. Since those initial demonstrations, the investigation of vasopressin has passed from a descriptive, systemic phase to a phase based on molecular investigations, in which the mechanism of expression and secretion of the peptide and the cellular basis of its actions have been elucidated.

But the advent of more sensitive and sophisticated methods of investigation has also shown that a restricted dual role for vasopressin is not a complete account of its functions. Arginine vasopressin is found exclusively in mammals, but it is a

member of a family of peptides with remarkably similar structures, known collectively as the neurohypophyseal (NHP) hormones. Vasopressin-like peptides have been detected in many species, and can be used to trace phylogenetic trees through structural changes in the molecule seen in different vertebrate groups.

Vasopressin has been shown to act on many tissue types. Heller, in a review covering early neurohypophyseal research, states,

“...it might be said that there is no endocrine organ whose active products exert so many different actions.” (1).

Even when this point of view was published much of the data on which this thesis is founded were not available. Since the initial descriptions of the influences of vasopressin on kidney tubules and blood vessels, vasopressin receptors and the effects of vasopressin have been described in the mammal at the cellular level in many different tissues – a list would include blood platelets, cerebral cortex, thymus, lateral spinal column, heart, lung, spleen, uterus, breast, liver, testis, pancreas, gastrointestinal tract and the adrenal gland. In addition, vasopressin has been shown to have a role in complex social behaviour and to enhance memory consolidation. This multi-functional aspect of vasopressin in different physiological systems raises the question of the possibility of an ‘original’ function, and an ‘original’ ancestral peptide. Vasopressin now has three recognised receptors; they are all members of the same super family of G-protein coupled receptors. As a starting point it seems logical to suggest that a single progenitor peptide existed, with a single cognate receptor and a single function. Subsequently a journey of mutation, with addition and/or change of function, has occurred until we arrive at the present situation, where vasopressin and

all the related peptides are involved in many different, discrete systems. This evolutionary process is a subject for much speculation.

It is also apparent that the hypothalamic magnocellular neurone is not the only site of vasopressin synthesis. Parvocellular neurones of the hypothalamus and cells in other tissues of both the brain and the periphery have been shown to fulfil a set of criteria that suggest that these cells are capable of the synthesis of biologically active vasopressin.

With this in mind, vasopressin, it can be argued, fulfils much more than an endocrine role. In other systems or tissues, it could be serving a paracrine or autocrine role. With such a wide range of effects, and with the differences in function between species and questions of physiological relevance in some species, it is almost impossible to present a complete and fully integrated description of vasopressin and its functions. But certain specific examples exist that lend themselves to a more detailed analysis and it would seem that a viable way to consider tissues involved is to distinguish between those tissues that only possess vasopressin receptors, and those tissues that apparently have the ability to synthesise biologically active vasopressin *as well as* functional receptors. Tissues that only express the receptors are under the 'control' of blood vasopressin levels, and so would conform to a consistent pattern of response within the physiological context of a rise (or drop) in these vasopressin levels. But those tissues that can produce a much higher local concentration can be considered as functioning within a closed system with little influence from or on the hypothalamo-neurohypophyseal system, albeit in co-operation with other regulators. In particular the adrenal gland has been the most

extensively studied example of this, and serves as a model for the examination of other putative sites of peripheral vasopressin synthesis and local action.

The participation of vasopressin in such closed systems should be considered as an important physiological aspect of function, because this peptide (along with others) can be considered to have evolved to the point where it is now integrally involved in many related processes. An intriguing aspect of this is the observation that vasopressin has been implicated as a growth factor in certain types of cancer. This effect could be considered as co-option of a normal physiological function - the vasopressin gene's participation in the growth of tumours should perhaps be viewed as a corruption of existing processes rather than a novel mechanism in itself.

A potential new role for vasopressin is discussed, that of a factor in the development of differentiated muscle tissue in the foetus. High levels of apparently specific vasopressin immunoreactivity (AVP-ir) have been described in samples of human foetal muscle, and work performed using rat myoblast cell lines suggest a physiological function for arginine vasopressin in the process of muscle development

1.2 Early neurohypophyseal hormone research

Looking at the literature on the study of vasopressin and oxytocin (OT), one is struck by the way the emergence of information about the peptide has mirrored the progression of technology, and the use of more sophisticated and sensitive techniques in science. The neurohypophyseal hormones have been the longest and most studied biological peptides, and their biological actions, structure and sources were the first examples of the description of such characteristics. Where vasopressin and oxytocin have led, so other peptide hormones have followed.

The first phase of the progression is the description of the systemic effects of vasopressin and oxytocin, culminating in the chemical synthesis of vasopressin. Oxytocin was the first peptide hormone to be synthesised *in vitro*, and vasopressin was synthesised soon afterwards (2, 3). This period of time is fascinating, as journal articles track the gradual gathering of information. Several reviews have been published about early neurohypophyseal peptide research, and much of the information presented here is drawn from these reviews.

After the report of the first synthesis of the peptide, there is a movement into the 'molecular' phase of study. This phase leads up to the present, and the knowledge we have about the processes involved in the transcription, translation, maturation, storage and secretion of vasopressin in the hypothalamo-neurohypophyseal system. Clearly, there is an unequal division of information in these two phases, and so the second phase of study is presented as a review of the information we have now, rather than a review of the steps in discovery from the synthesis of vasopressin to the time of writing.

The study of vasopressin started with the recognition in the eighteenth century by the Venetian anatomist Santorini that the anterior pituitary gland was not connected to the pituitary stalk (4). After this distinction between the 'pars anterior' and 'pars posterior', Soemmering (in 1778) proposed an alternative to these names - the hypophysis - and the term neurohypophysis was born. Following this addition to the nomenclature, little progress (in the form of published reports) was made for over a century, something Heller tentatively explains by saying

“...when studied with ordinary methods of fixation and staining, the posterior lobe has a rather nondescript appearance.” (4).

But study of the pharmacology and physiology of vasopressin started with the publication of a communication by Oliver and Schäfer in 1895 (5). This study reported that extracts of fresh whole pituitary gave rise to an elevation of blood pressure when injected into anaesthetised mammals. Three years later, Howell confirmed that the vasopressor-causing principle was in the posterior pituitary by showing that anterior pituitary extracts had little or no effect on blood pressure (6). Schäfer and his colleagues observed diuresis and expansion of the kidney during and after their experiments, and for many years the consensus opinion was that neurohypophyseal extracts were actually diuretic. That the reverse was true (in situations where the animals urine did not have a higher osmotic pressure than plasma) was demonstrated simultaneously by “two venturesome clinicians” (7), Von den Velden and Farini. Both of these investigators showed that injecting extracts of posterior pituitary glands into patients secreting large volumes of weak urine reduced this diuresis (8, 9). Von den Velden took initial observations one step further and showed that the extract also reduced water diuresis in healthy subjects, establishing that the active principle had an effect in the normal physiological state. This observation was repeated by others, and was also shown to occur in a similarly treated rabbit.

Demonstration of the effect of anti-diuresis by posterior pituitary extract was followed by a description of the mechanism of anti-diuresis, by Starling and Verney. They isolated a kidney in a “heart lung” perfusion circuit – it produced dilute urine.

Extract from the posterior pituitary reduced the amount of urine and increased the concentration of the urine produced. Further, when a head was included in the circuit a similar effect was seen. When the neurohypophysis was removed from the head prior to the set up of the circuit, anti-diuresis was not observed (7). This was the most rigorous proof thus far that the active anti-diuretic principle came from the posterior pituitary, albeit on the understanding that these experiments have probably never been repeated by anyone else (7). Later, Verney showed that the anti-diuretic effect could be elicited by causing a small rise in plasma osmotic pressure using hypertonic saline (4, 7).

Bargmann used staining of the structure of the hypothalamus and posterior pituitary to persuade a sceptical mammalian endocrinologists that neurohypophyseal hormones were produced in the hypothalamus (10).

Four biological actions ascribed to posterior pituitary extracts were eventually established – uterus contraction (an oxytocic action), milk ejection (a galactagogic action), increase of blood pressure (a vasopressor action) and renal urine/water reabsorption (an anti-diuretic action). But the nature of the source of these activities was still not clear. Abel and co-workers postulated a “mother molecule”, in which all four effects were observed to be exerted by the same protein, whereas Dudley was equally convinced of the existence of distinct principles (11). Van Dyke described the isolation of a protein of molecular weight 30,000 Dalton (Da) that possessed all four activities, whereas Du Vigneaud showed that two small peptides could be isolated which split the activities. Despite the fact that Du Vigneaud had determined the structure of vasopressin and synthesised it along with oxytocin, it was still not clear which of the two possibilities was the genuine physiological state

and which was produced as experimental artefact. Chauvet and colleagues showed that the Van Dyke protein could be dissociated into a heavier inactive portion and the smaller active portion. Moreover, the two could be re-complexed in a stoichiometric mixture in the right conditions (12). The term neurophysin was suggested for the inactive protein element, which was later shown to contain two homologous components that could be distinguished by their N-terminal sequences (13). The Van Dyke complex was prepared from posterior pituitary glands from a variety of mammals, and from the relative amounts of the two pairs of peptides and the neurophysins it was suggested that they were derived from two common precursors (14). This was shown to be correct by the use of molecular biology techniques to find the entire amino acid sequences of both pro-vasopressin and pro-oxytocin (15, 16).

1.3 Magnocellular neurones in the hypothalamus – a site of vasopressin synthesis and release

The classically described site of vasopressin and oxytocin gene expression is in the hypothalamus. The hypothalamus, together with the posterior pituitary and anterior pituitary glands comprises a fundamental endocrine structure. The hypothalamo-pituitary axis and the basic unit of neurohypophyseal peptide production - the magnocellular neurone - have emerged as paradigms for polypeptide synthesis and secretion, and the study of the processes involved in the mature expression of this phenotype proceeds on that basis (17). It is a feature of eukaryotic cells that they possess an ordered and efficient production pathway for the synthesis and trafficking of proteins, and this is shown to full effect by these neurones.

Essentially they are neuroendocrine cells, capable of both the propagation of action potentials and the secretion of hormones. These neurones possess large cell bodies responsible for the production of vasopressin and oxytocin, and are found in two paired groups (or nuclei) in the anterior hypothalamus, the supraoptic (SON) and paraventricular (PVN) nuclei. Virtually all magnocellular neurones in both nuclei project to the posterior pituitary. The SON contains three times the number of cells as the PVN, but they both contain the same proportion of vasopressin- or oxytocin-containing cells. Vasopressin and oxytocin are only very rarely found in the same cell (18). The SON is typically much more homogenous than the PVN. The PVN contains many subnuclei. As well as contributing magnocellular neurones to the posterior pituitary subnuclei include some parvocellular neurones that whilst they also synthesise vasopressin, project to other structures in the brain.

Axons of magnocellular neurones lead down the pituitary stalk to the posterior pituitary gland. This gland is the site for direct entry of neuroendocrine substances into the systemic circulation. The nerve terminals are situated immediately adjacent to capillaries. Excitation of the neurone leads to fusion of secretory granules with the plasma membranes and the emptying of their contents into the extracellular or perivascular space.

Vasopressin and oxytocin are rarely found in the same cell (18). The reason for this is not clear, but it appears that there are specific factors involved in the phenotypic determination and growth of the neurones at the onset of their development (19, 20). Vasopressin immunoreactivity can be detected in the posterior pituitary early in foetal development (21, 22).

Control of gene expression operates on several levels. Osmotic stimuli such as dehydration or salt loading result in increased transcription (and thus AVP messenger ribonucleic acid (mRNA) abundance) and an increase in poly-A tail length on the transcripts. The precise machinery behind this control has not yet been fully elucidated (23).

Vasopressin, an amidated nonapeptide, is initially translated as a preprohormone. It is derived from this much larger translated precursor of 145 amino acids. The vasopressin gene has been identified and sequenced in many mammalian species. The gene is located on chromosome 20, and is linked closely to the oxytocin gene. Divided into three exons, it is punctuated by two introns (24, 25). The first exon encodes a signal peptide (ensuring correct trafficking of the primary translated product), vasopressin itself, a bridging tripeptide, and the N-terminal sequence of the protein called the neurophysin. The second exon encodes the main body of the neurophysin (up to amino acid 76 of 93/95 amino acids). The third exon encodes for the C-terminal sequence and a small glycopeptide of 39 amino acids, also known as vasopressin-associated glycopeptide (VAG) or copeptide. As translated mRNA is transported into the endoplasmic reticulum, the signal peptide is cleaved from the peptide. Processing in the Golgi apparatus of the smooth endoplasmic reticulum packages the prohormone into secretory vesicles for transport through the axon to the posterior pituitary. During this transport the prohormone is subjected to enzymatic changes that result in the final product of alpha-amidated nonapeptide. This maturation involves a cascade of at least 4 enzymes that operate at the tripeptide signal between the peptide and the neurophysin and the single amino acid connecting the neurophysin and the glycopeptide – shown in Figure 1.1. First, a dibasic

The vasopressin gene DNA is transcribed and translated to generate the translated preprohormone

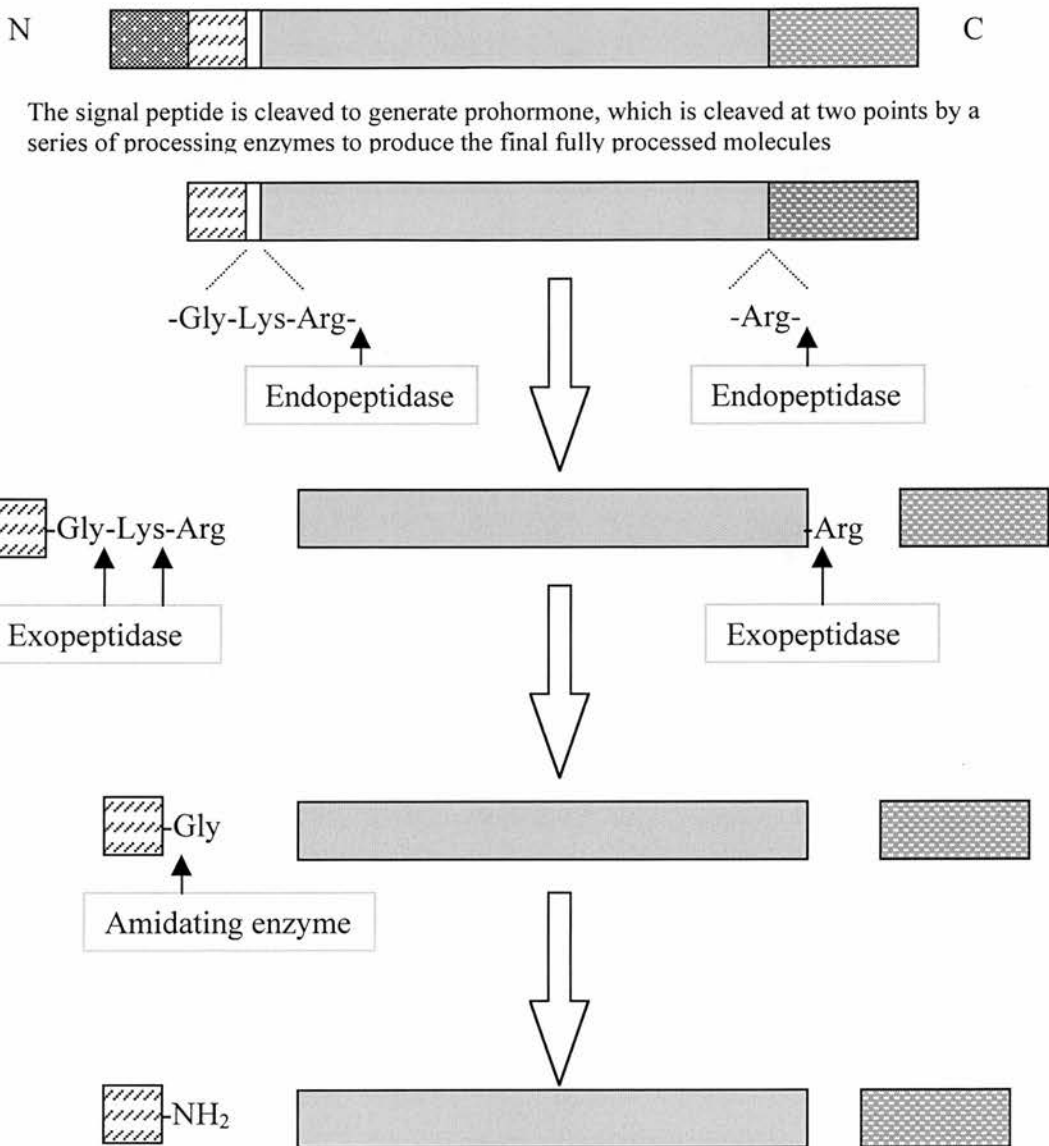
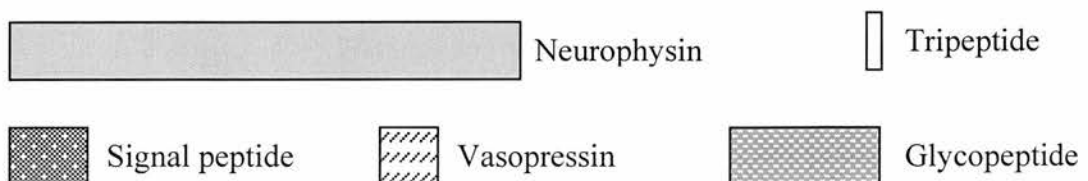


FIGURE 1.1 Processing events in the synthesis of mature vasopressin
(Adapted from 26)

Vasopressin is synthesised with neurophysin and a glycopeptide, together with trafficking signals directing the mature products to secretory vesicles.



endopeptidase cleaves the Lysine (Lys)-Arginine (Arg) bond, separating the neurophysin and creating a twelve amino acid intermediary product. Next a carboxypeptidase B-like enzyme (carboxypeptidase E or H) cleaves these two basic residues, leaving a decapeptide. Then peptidyl-glycine alpha-amidating monooxygenase (PAM - a single molecule which has two enzyme functions) acts on the glycine residue at position ten to leave a peptidylhydroxyl glycine, which is in turn split off by the second portion of the same enzyme which acts as a lyase enzyme, resulting in the amine group attached to the now mature peptide and free glyoxylic acid (26). This processing is 95% complete before the arrival of the secretory granule at the terminal bud, so that only mature peptides are found in the posterior pituitary (27).

In mammals, vasopressin originating in the posterior pituitary gland is involved in the homeostasis of the osmotic pressure of body fluids. Osmolality is maintained at a constant level on an hour to hour basis, and severe alterations caused by a lack of control by vasopressin exerted by can lead to trauma caused by changes in the volume of the brain, and even death (28). To achieve the control of plasma osmolality, vasopressin must respond to small changes. Cells sensitive to very small changes in extra cellular fluid osmolality (osmoreceptors) present on the anteroventral aspect of the third ventricle and outside the influence of the blood brain barrier (29, 30) are thought to be responsible (as little as a 2% rise in plasma osmolality can cause a drop in urine output (31)) by acting as stimulus for the release of vasopressin.

In addition, blood volume depletion of more than 10% and rapid drops in blood pressure will also cause a rise in plasma vasopressin. Volume responses are

initiated by receptors in the left atrium, and it is thought that pressure changes are detected by baroreceptors in the carotid sinus (32). Both osmotic and non-osmotic stimuli cause the release of vasopressin via neural mechanisms. Action potentials are propagated from the receptor cells to the magnocellular neurones causing a depolarisation of the axon terminals in the posterior pituitary gland. This in turn causes the influx of calcium ions (Ca^{2+}) which brings about the exocytosis of vasopressin - the fusion of neurosecretory vesicles with the cytoplasmic membrane causing the contents to be released into the extra cellular space. Magnocellular neurones terminate adjacent to capillaries which allow the contents of the vesicle to be released directly into the blood stream (33).

1.4 Signal transduction of vasopressin

All actions of vasopressin are caused by binding of vasopressin to receptors present in the surface membrane of the targeted cells. The receptors activate cytoplasmic second messenger cascades to transduce the initial signal at the molecular level. Three recognised receptors have been identified; the V2 receptor, and two subtypes of the V1 receptor - the V1b receptor and the V1a receptor. The V2 and the V1-type receptors activate different second messenger cascades, and so expression of more than one type of receptor will, upon activation by vasopressin, lead to different effects being caused, with different time courses, in the same cell. Originally the receptors were identified as being two separate types, as the different types have different affinities for analogues (34), and the V1-type receptors were further subdivided by their pharmacological affinities for different analogues of AVP (35). Subsequently, molecular biological techniques have confirmed that although

they share a common structural motif - neurohyphyseal receptors collectively have a 35-60% amino acid sequence homology, and can be considered a subfamily of the seven transmembrane span G-protein coupled receptors (GPCRs) (36, 37) - they are different proteins.

1.4.1 V2 type receptor

The vasopressin receptor that mediates the classically recognised antidiuretic hormone action has been designated the V2 receptor (34). Binding of vasopressin to this receptor results in water re-absorption in the collecting duct of the kidney. The receptor activates adenylyl cyclase, leading to the production of cyclic AMP (cAMP), which in turn activates protein kinase A. This enzyme causes the increased insertion of aquaporin molecules, water channels through which osmotically driven water molecules can pass from the collecting ducts into the interstitium (38). The human receptor has been localised to the X chromosome (39). It is predicted to be a 371 amino acid protein with a molecular weight of approximately 40.2kDa (40). The rat receptor is predicted to be a 370 amino acid protein with a molecular weight of 40kDa (41).

1.4.2 V1-type receptors

V1-type receptors are activated by the binding of the hormone to the cognate region on the receptor. Accumulation of cAMP is not observed after V-1 type receptor activation (42). Instead, these receptors promote the exchange of GDP for GTP at the binding sites of G-proteins, facilitating the G-protein catalysed activation

of phospholipases C, D and A2 - enzymes capable of breaking down phospholipids into constituent molecules (43).

Phospholipase C catalyses the breakdown of inositol phospholipids into inositol phosphates - most commonly inositol 1,4,5-triphosphate (IP₃) - and 1,2-diacylglycerol (DAG) (42). IP₃ causes release of calcium stores in the endoplasmic reticulum, resulting in a transient increase in intracellular calcium concentration. DAG activates protein kinase C, an enzyme that activates many proteins by phosphorylation of specific residues in target proteins involved in cellular responses to hormone signals (44, 45). The Na⁺-H⁺ exchanger is also activated in certain cells (35, 46).

Phospholipase A2 produces arachadonic acid, which in turn is used to generate prostaglandins and thromboxanes (46), as well as enhancing movement of calcium ions into the cell from outside and from calcium stores within the cell (35).

Phospholipase D catalysed breakdown products include phosphatidic acid, which can alter the activity within the Ras pathway, or when broken down further produces DAG (35).

1.4.2.1 V1b receptor

Vasopressin stimulates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, and therefore participates in the stress-induced ACTH surge. The transduction of vasopressin binding to the V1b receptor is coupled to different G-proteins depending on the concentration of receptors resident in the membrane. At low concentrations the receptor couples to the G_{q/11} subtype. At higher

densities the receptor can interact with both the G_i and G_s , and thereby activate several different signalling pathways (42).

The human V1b receptor is predicted to be a 424 amino acid protein with a molecular weight of approximately 47kDa (47, 48). Northern blot analysis by this group (48) failed to find evidence of the transcript in any human tissues apart from the pituitary gland (site of ACTH release), the tissues tested being heart, brain, lung, placenta, skeletal muscle, pancreas, kidney, and liver. However, a faint signal was present in mouse tissues, those tested being hypothalamus, brain, adrenal, pancreas and colon (49)

Rat tissues have also been analysed by the use of reverse transcriptase-polymerase chain reaction (RT-PCR) and found V1b expression in the thymus, heart, lung, spleen, kidney, uterus and breast. This report also stated that V1b mRNA was not detected in the liver, adrenal gland (they do not specify whether cortex or medulla was used), seminal vesicles and testis (50). RT-PCR studies of the pancreas revealed a positive signal for this receptor (49).

1.4.2.2 V1a receptor

The V1a receptor designation was created as a consequence of the recognition of the V1b subtype and its different pharmacological profile (51). V1a receptors are coupled to a G-protein (52) – primarily the $G_{q/11}$ subtype (42) but also the G_i subtype (53). Secondary nuclear signal mechanisms that are activated by binding of vasopressin to the V1a receptor include the induction of immediate-early response genes expression, causing protein synthesis which culminates in hypertrophy and increased protein content of the cell (54). Other recognised actions

include blood vessel constriction, liver glycogenolysis, platelet adhesion, stimulation of adrenal gland secretion, and certain brain functions.

The gene (a single copy) for the V1a receptor has been localised to chromosome 20 (55). cDNA sequences for the human receptor predict a 418 amino acid segment of the receptor of approximately 47kDa molecular weight (56). cDNA sequences for the rat receptor predict a 394 amino acid segment of approximately 44.2kDa molecular weight (41). However, reports of the molecular weight of the receptor using other methods differ, and it may be that these estimates are affected by dimerisation and glycosylation of the receptor. One of the first estimates of the weight of the rat V1a receptor using migration in sucrose density centrifugation was 83kDa (57); double what might be expected. Whilst Western blotting analysis led to an estimate of 44.2kDa in rat tissues (58), it is clear that the receptor is glycosylated in its fully matured form. A photoprobe with high affinity for the receptor was used to produce an estimate of 53kDa for the rat receptor, and treatment with a deglycosylation enzyme reduced this estimate to 43kDa (59).

The V1a has four consensus sites that may potentially be glycosylated, and three of them are reported to be used in translation and processing of the rat V1a receptor (37). Interestingly though, these studies also revealed that the glycosylation was not important for the function of the receptor. Mutated receptors lacking the glycosylation were not expressed with lower efficiency, bound analogues of AVP with the same affinity and specificity as the wild type receptor, and participated in cell signalling cascades fully. However, V1a receptors show variation in their affinity for different ligands between species, and so this may not be the case for all species.

Using Northern blot analysis, mRNA coding for the V1a receptor has been demonstrated in the liver, heart, kidney and skeletal muscle in human tissues (55). The Northern blot analysis of rat tissues obtained positive results in the liver, kidney, spleen, and testis (41, 60). But the expression of the V1a is species specific and has a developmental aspect to it. Whilst the commonly studied rat expresses ample V1a receptor in the liver, human (61), rabbit (62), and sheep (63) liver have been shown to show almost a complete lack of these receptors, and their participation in regulating hepatic function in these species has been questioned.

Of relevance to this thesis is the specific developmental expression of the V1a receptor. In the rat liver, V1a receptor is not seen before birth, and the authors point out that this means that the liver is not a target for vasopressin at this stage of development (although vasopressin is detected prenatally) (64). After birth, when the now independent rat is solely responsible for control of glucose levels, expression of V1a mRNA and translation of this message into functional receptor is rapid. In rat testicular myoid cells, V1a receptor is not seen before puberty, but is present in adult cells where it potentially plays a role in regulation of testicular function (65). These two studies serve to establish a principle, that expression of the V1a receptor is tightly controlled in order to allow specificity in the action of AVP. On the evidence of these two studies, there is not a case to be made for proposing that V1a receptor is generally expressed in many tissues types with no specific role in the function of that tissue.

1.5 Evolution of vasopressin and cognate receptors

The effects of vasopressin are widespread, and are mediated by the receptors discussed in Section 1.4. Oxytocin, and the single receptor type that recognises it, are not described in this section, and this should reflect the point that the neurohypophyseal peptides are a group of distinct peptides with distinct actions, that occur through the mediation of distinct receptors.

Diversity in the neurohypophyseal hormones in different species was demonstrated before the molecular structure of any peptide was actually known. Heller showed this by comparing the relative activities of standardised amounts of posterior pituitary extracts in bioassays (66). The fact that extracts from different animals had different potencies in the same conditions meant that the relevant components of the extracts had to be structurally different in some significant way that a receptor in the bioassay recognised the molecules with different affinities and potencies. The ability to synthesise peptides and in particular the description of vasopressin and oxytocin was a key to the further investigation of these related peptides. In some cases, peptides have been speculatively synthesised before their presence has been demonstrated in an organism. Sawyer recounts an instance of this (67). Katsoyannis and du Vigneaud had synthesised a hybrid peptide consisting of the ring of oxytocin and the tail of vasopressin, and named it arginine vasotocin (AVT) (68). Munsick and colleagues (of which Sawyer was one), studying a peptide contained in extracts from chicken neurohypophysis found that the peptide behaved exactly like the synthesised peptide when subjected to chromatography and chemical analysis (69). This was later confirmed when Heller and Pickering chemically characterised and identified AVT in non-mammalian pituitary glands (70).

To date, thirteen neurohypophyseal hormones have been identified (71). The conservation of amino acid sequence and, therefore, structure is remarkable (see Table 1.1). Within the limits of larger requirements for intrinsic stability of the molecule, the variation of the peptides must also be limited by the requirement that the peptide fits to a specific receptor. (If the variation is large so as to not be recognised by the receptor, it must be a non-functional mutation and presumably not likely to be of any improvement on the previous peptide.) They are all nonapeptides, and they all have a disulphide bridge (involving two cysteine residues) between positions 1 and 6. The amino acids at position 2 and 3 are always hydrophobic, and those at 4 and 5 are always polar. Of the three tail amino acids, 7 and 9 are invariant, apparently supplying a protective conformation against degradation and structural rigidity to the molecule (11). The variations are only seen at amino acid positions 3,4 and 8. Three dimensional structural predictions based on deaminoxytocin show how substitutions at these positions can change the specific identity without radically modifying the general conformation (72). For example, vasopressin-like peptides have a basic residue (Arg or Lys) at position 8, whereas oxytocin-like peptides virtually always have a neutral aliphatic hydrophobic residue at this position. The biological significance of such substitutions are clearly shown by the transformation of AVT to AVP. Replacement of isoleucine by phenylalanine at position 3 gives rise to a peptide that acts only on the kidney and vascular receptors, but not on uterus and mammary gland receptors (as AVT also does).

As a result of this stability within the small confines of a nine amino acid peptide, they are limited in their value as a basis for delineating phylogenetic relationships established by other methods. Much better candidates for such a

	NH ₂ -	1	2	3	4	5	6	7	8	9	COOH
Basic structure		Cys	Tyr	•	Gln	Asn	Cys	Pro	•	Gln	
		└──────────────────┘									
Arginine vasopressin (AVP)				Phe						Arg	
Lysine vasopressin (LVP)				Phe						Lys	
Arginine vasotocin (AVT)				Ile						Arg	
Oxytocin (OT)				Ile						Leu	

TABLE 1.1 Chemical structure of mammalian neurohypophyseal hormones

The backbone of the peptides, a nine amino acid peptide consisting of a six amino acid ring (linked by cysteine residues) with a three amino acid tail, is a consistent motif not only in mammals but all families shown to express forms of this peptide. The structural similarity of the peptide hormones provides information as to the evolution of function of the hormones, and demonstrates the remarkable specificity of receptors to particular peptides to discern them from others present in the body.

- - indicates positions of amino acids substitutions

Arg = Arginine	Gln = Glutamine	Phe = Phenylalanine
Asn = Asparagine	Ile = Isoleucine	Pro = Proline
Cys = Cysteine	Leu = Leucine	Tyr = Tyrosine
Lys = Lysine		

process are proteins such as mitochondrial cytochrome *c*, where there is sufficient significant change between species and orders to distinguish them and construct time-line relationships between them on the basis of the enzymes' primary structures (73).

But changes have occurred to the neurohypophyseal peptide gene throughout evolution. In fresh water *Hydra*, which possess the most primitive nervous system known and does not have a feature comparable to blood, the presence of not only vasopressin/OT-like immunoreactivity but also gastrin/cholecystokinin, Substance P, neurotensin and bombesin/gastrin-releasing peptide-like immunoreactivity have been demonstrated. This suggests that peptides played a role as neurotransmitters long before being 'recruited' as hormones in 'more evolved' species (11). AVP/OT-like immunoreactivity has been found in various classes of invertebrates (74-76). Their functions are largely unknown. However, there is no evidence for the co-existence of both vasopressin-like and oxytocin-like immunoreactivity in the same invertebrate species. Thus, a single copy of the gene is present in the genome at that point in phylogenetic development.

Neurohypophyseal peptides are found in all vertebrates. The most 'primitive' vertebrates, the cyclostomes, apparently only express a single peptide, AVT. This seems to suggest that AVT is the original ancestral gene of both arginine vasopressin and oxytocin, which are apparently the most recent transformations of the neurohypophyseal peptides. This also suggests that the existence of the two different peptide types in the same organism in subsequent lines of phylogenetic development is the result of a gene duplication event (77). Such events are central mechanisms in the generation of functional diversity and shifts or additions of function (78). Gene

duplication is also the mechanism by which the subtypes of vasopressin receptor were most likely to have been generated (11).

The evolution of the peptides and their receptors seems to be an example of co-evolution (79). All species that express a neurohypophyseal (or NHP-like) peptide also express an associated receptor that has significant sequence homology with other member of the G-protein linked receptor group but is strongly specific for that particular peptide. The phylogenetic point at which this duplication occurred is not clear. Similarly, the first physiological function of the vasopressin precursor in vertebrates can only be speculated on. Since AVT affects cardiovascular function in non-mammalian vertebrates, and vasotocin receptors are distributed throughout the cardiovascular system in these animals, Sawyer suggests that the most primitive functions of vasotocin are related to cardiovascular regulation (77).

Darlison and Richter examined many examples of multiple receptor types in a single genome and concluded that receptors evolved as a result of mutations of the peptide; a subsequent change in the receptor then conferred specificity to the initial change in the structure of the peptide (80). Viable mutations would have to retain some affinity for the original receptor, and receptor mutations would have to similarly be able to recognise one or both of the peptides. But through this process, and that of gene duplication, different responses could be mediated by different receptors responding to the same peptide.

But the principle is established that the gene coding for the original peptide, the processing enzymes that are able to produce a mature translation product, and a receptor specific to the peptide, existed before the invertebrate/vertebrate divergence. That the precursors to vasopressin had functions of physiological significance before

the emergence of the neurohypophysis as an anatomical structure also establishes a principle central to this thesis. It is perhaps better to use the phrase ‘diversity of action’ rather than the word ‘function’, for at a very simplistic level the function of the neurohypophyseal peptides is to bind to their respective receptors, and the function of the receptors is to transduce the event of binding into a biochemical signal with the cell. This is invariant throughout the range of examples of the peptides. The change that is manifested is in the tissues and responses that the peptide and receptor mediates. It is proposed that the ancestral vasopressin-like molecule was a neurotransmitter in the first instance of the ability of a peptide to exert a biochemical change (76). At the other end of the phylogenetic tree, vasopressin (lysine and arginine) is the antidiuretic hormone and oxytocin is involved with parturition and the milk let-down reflex. Along the way, the peptides have undergone a change in the site of synthesis, and therefore a change in their mode of action, from local neurotransmitter to endocrine hormone. It should therefore be obvious to state that there has been a change in the tissues the peptides exert an effect on, and a change in the effect they cause.

Sawyer speculates that the presence of vasopressin receptor in the blood vessels might serve no physiological function, on the grounds that maximal antidiuresis is achieved at plasma concentrations of vasopressin well below those that have an effect on blood pressure, and the receptors are “merely vestigial reminders of our evolutionary history” (77). Whilst perhaps not agreeing with the example chosen, the point is clear – there are circumstances in which *in vitro* effects of vasopressin are seen, but at concentrations that cannot be matched by any observed in samples of blood plasma. Such an *in vitro* observation can be viewed in

two ways, as an artefact of the *in vitro* system, or as a genuine phenomenon. Just as the levels of blood vasopressin are not high enough to cause the *in vitro* effect *in situ*, so the system exerting the control might not be seen when examining whole body preparations. That is to say the action is paracrine or autocrine in nature, and concentrations required to cause the effect are not reflected blood plasma levels. In short, observations like those of Sawyer argue for the synthesis of vasopressin in areas other than the hypothalamus. This is entirely in keeping with the origin of this molecule – it was a non-hypothalamic regulatory peptide, and the processing machinery required to generate active peptide should be able to operate normally in such situations. Examples of proposed alternative sites of synthesis are described below.

There should, therefore, be no surprise to find AVP involved in the process of cancer. Viewing vasopressin purely as a regulatory hormone denies the progression in development of action that leads to the arrival at its present roles. Examples of vasopressin acting as a growth promoter in tissues are common, and some of these are discussed. One way of viewing causes of cancerous growth is the result of inappropriate and uncontrolled expression of growth factors in tissues, rather than the occurrence of an aberrant and novel function (81). The involvement of vasopressin in certain types of cancer could be viewed as a example of this uncontrolled expression, a recapitulation of the ability of the peptide to exert an effect that predates the one it is predominantly associated with in mammals (82-84).

1.6 Alternative sites of synthesis and local action of vasopressin

It has been stated (85) that the first demonstration of extrahypothalamic vasopressin immunoreactivity was by Wathes *et al* in the human ovary (86). However, earlier reports of the detection of neurophysin-like material in the uterus and pineal gland predate this as an example of NHP peptide localisation in extrahypothalamic areas (87). The significance of these earlier reports were often downplayed in the concluding comments, perhaps due to some resistance to the idea that NHP peptides could be anything other than *the* neurohypophyseal peptides (a good example of this attitude is a later article entitled “Is the evidence for ectopic antidiuretic hormone watertight?” (88)).

Reports of concentrated vasopressin immunoreactivity are often attributed to sequestration of the peptide by specific factors. An example of uptake of vasopressin to produce local AVP concentrations above that of circulating plasma levels is that seen in the pituitary and pineal glands (89). Another often cited example of sequestration of AVP is that carried out by blood platelets. These cells are involved in blood clotting and wound repair processes, and are derived from mononuclear cells. Approximately 90% of circulating AVP is ‘associated’ with platelets (90). AVP immunoreactivity extracted from platelets was seen to behave in exactly the same way as peptide extracted from the posterior pituitary – it diluted in parallel to AVP reference standard in a radioimmunoassay (RIA) of the peptide, and it had the same retention time on a high performance liquid chromatography (HPLC) column as synthetic peptide. Tellingly, a bioassay was performed, and the extracted AVP was biologically active in the rat assay for antidiuretic activity (91).

When exogenous AVP was infused into human subjects, a significant increase in both plasma and platelet-associated AVP was found. This increase occurred in a consistently proportional manner to provide an equation for the distribution of immunoreactivity (plasma AVP = $2.48 + 0.11$ platelet AVP). Additionally, patients with idiopathic and post-surgical diabetes insipidus were seen to possess negligible platelet AVP. All these data suggest that the AVP associated with platelets is taken up from plasma and originates from the hypothalamus. Despite there being a similar blood plasma concentration of oxytocin, no oxytocin appears to be associated with platelets.

Berretini described a specific saturable binding of ^{125}I -AVP to human platelets, which showed specificity characteristic of V₂-type receptors (92). However, when Vittet and colleagues compared the binding of ^3H -AVP to membranes prepared from human platelets and rat liver and kidney, it was found that the binding characteristics of platelet preparations were comparable to those of liver membranes but not the kidney membranes (93). This was backed up by the affinities the preparations showed for different vasopressin analogues. Thibonnier confirmed the receptor on platelets was similar to the V_{1a}-type receptor (94).

Vasopressin was shown *in vitro* to promote the aggregation of human platelets (93). Binding of vasopressin with platelet membranes stimulated the phosphorylation of two calcium-dependant proteins (94). The peptide has been shown to activate platelets, leading to adhesion, aggregation and secretion, but originally this was seen only at concentrations of vasopressin many orders of magnitude above the free plasma concentrations for normal humans (94, 95). However, using flow cytometry and monoclonal antibodies to identify specific

proteins, Wun and colleagues demonstrated activation of platelets after incubation with physiological concentrations of vasopressin (95). It is speculated that platelet binding of vasopressin may play a role in the rapid clearance of the hormone from the circulation, or more plausibly that this uptake may indicate a role in modulating platelet function. Vasopressin is released in situations of stress, and the activation of platelets in situations which potentially involve acute blood loss is consistent with a role for vasopressin in the mediation of stress responses. Thus, vasopressin can be seen to be concentrated from sources containing normal, low levels of the peptide, perhaps to achieve the concentrations required to activate the second messenger cascade of the receptor.

Two reviews authored with the aim of discussing the idea of extrahypothalamic NHP peptides strike a note of caution at inferring physiological significance to observations of direct vasopressin and oxytocin immunoreactivity in peripheral tissues (85, 96). Murphy and co-authors assert that the evidence of a positive result by Northern analysis should not be interpreted blindly as proof of an active, translated product. The example supplied is the positive result gained in experiments on testis tissues. Transcript isolated from testis tissue was shown to lack the vasopressin encoding exon, and although small amounts of the full transcript could be found, the authors concluded that with evidence available the levels of transcript were too low to be of undoubted physiological significance.

The many methods used in the investigation of peripheral and non-magnocellular neuronal synthesis of vasopressin are also reviewed. Despite the reservations expressed by the authors, the vast majority of the studies cited do not use molecular biological techniques as the primary source of data. Instead,

immunological methods of detection are employed; usually immunohistochemistry or radioimmunoassay. More sensitive methods that could be used, such as *in situ* hybridisation, Northern blotting and use of RT-PCR, are only employed in studies following initial immunological studies.

A crucial point is this conclusion:

“Only in one non-pathological case, the expression by the bovine ovary of oxytocin, does peripheral expression result in an elevation of plasma hormones levels, which could have systemic effects and be of physiological significance.... ...for all other instances of extrahypothalamic VP and oxytocin gene expression, we are reduced to postulations involving subtle autocrine or paracrine functions” (96)

This conclusion seems to seek to emphasise the position that only circulating levels of vasopressin have “physiological significance”, only validating those functions that are regulated by circulating vasopressin itself. It is hoped that in the examples shown below, it is clear that basal plasma levels of vasopressin have no relevance to the action of vasopressin at the local level, at least in the periphery, where vasopressin is seen to act as a paracrine or autocrine hormone and supplies levels of vasopressin that cannot commonly be created in the circulation.

The point is well made, however, that there are certain conditions that must be fulfilled for a cell or tissue to express an active vasopressin molecule, and one that functions in the local environment. Essentially they are the same requirements embodied by the expression of vasopressin in the magnocellular neurones of the hypothalamus. Transcription factors must be present in order to promote gene transcription to provide a full mRNA that can be translated into the peptide itself, the neurophysin and the VAG. Processing enzymes must be present to produce an active final product from the translation. Beyond that, it would seem desirable that the peptide be packaged in a way that it can be released, that is to say within a secretory

vesicle. Also, the tissue should be shown to possess functional receptors to vasopressin in order that this signal be transduced and exert an effect. It would seem logical to suggest from these criteria that cells equipped to synthesise other peptides are better candidates for sites of vasopressin synthesis than those that are not. Such a theory is in adherence with the contention that polypeptide synthesising cells are derived from the same embryonic layer, the neural crest (97). Pearse calls these cells APUD cells (Amine Precursor Uptake and Decarboxylation). It is a compelling theory, but evidence to demonstrate exceptions is widespread (98). Debate on this matter is ongoing. Until the matter is more satisfactorily resolved, it cannot be discussed in the context of description of tissues that are proposed to synthesise vasopressin beyond agreement with the proponents of a wider source of endocrine cells. It seems better to say that these cells share biochemical features that are acquired through differentiation rather than embryological programming (99).

The cases of non-magnocellular neuronal production of vasopressin are reviewed below. Where possible, the criteria listed above are addressed in the case of each tissue. As will be seen, however, there are few tissues in which a full investigation has been carried out. The question of the processing enzymes being present in cells is rarely addressed. As a result, there is clearly a division between neuronal cells (that are assumed to possess the requisite enzyme through their phenotype) where the vasopressin presumably acts as a neurotransmitter and so stimulates structures with well defined roles, and other tissues (whatever designation the cells within tissues that produce vasopressin eventually receive) where the role of the peptide is less clear beyond activating resident receptors, and so the two areas of observation are dealt with separately.

1.6.1 Vasopressin in brain tissue

1.6.1.1 Vasopressin within the hypothalamus

Within the hypothalamus itself, parvocellular neurones situated in the paraventricular and suprachiasmatic nuclei, which project to a number of regions in the brain, synthesise active vasopressin (100). Some of these neurones terminate in the median eminence, where vasopressin is released into the portal system that drains into the anterior pituitary gland. Vasopressin from these neurones acts as a releasing factor for adrenocorticotrophic hormone (ACTH). Thus, vasopressin is intimately involved with the stress response. Other neurones are found ending in the medial septum where vasopressin is thought to play a role in antipyresis. (101, 102). Neurones terminating in the spinal cord are thought to be involved with regulation of blood pressure (103). Vasopressin originating in the parvocellular neurones of the suprachiasmatic nucleus is also thought play role in circadian control (104).

1.6.1.2 Vasopressin in brain tissue beyond the hypothalamus

Vasopressin immunoreactivity has been demonstrated in several regions of the brain. Vasopressin immunoreactivity and vasopressin mRNA have been demonstrated in the medial amygdala and bed nucleus of the stria terminalis. Axons from these nuclei project to various regions of the brain such as the lateral septum, diagonal band of Broca, locus coeruleus, amygdala and ventral hippocampus (105). The lateral septum mediates the effect of vasopressin on body temperature, the amygdala and ventral hippocampus have roles in learning and memory. Vasopressin

has been shown to facilitate memory consolidation and retrieval in rats (106, 107). In humans vasopressin was shown to be a contributing factor in the improvement of memory, a function that essentially relies on hippocampal function.

Vasopressin has also been demonstrated in the pineal gland of the rat (108), sheep (108), and cow (109). Neurophysin-like immunoreactivity and activity was detected in the human pineal gland (87). In the rat, the levels of pineal vasopressin were shown to vary according to the season, with a high in the summer months (108), a variation not seen in hypothalamic levels of vasopressin mRNA or pituitary levels of vasopressin (110). Vasopressin mRNA has been demonstrated by in situ hybridisation histochemistry in cells in the pineal gland (111). The active peptide generated in this gland probably has some intraglandular activity because V1a-type receptor mRNA has been detected in adult male rat pineal cells (112). Vasopressin binding was also demonstrated in pineal glands from the rat, which was shown to be saturable and specific to the V1a receptor subtype (113). This study also demonstrated that vasopressin potentiated noradrenergic secretion of melatonin (and this was inhibited by a V1a antagonist). The authors concluded that vasopressin could be a locally synthesised modulator of rat pineal synthetic activity exerting an influence through the V1a receptor subtype.

1.6.2 Vasopressin in peripheral tissues

It is interesting to note that there are many descriptions of neurohypophyseal peptides in peripheral tissues, and in many of these cases any vasopressin immunoreactivity is accompanied by oxytocin immunoreactivity. The relative abundance of the two peptides is stated where possible, as is any proposed function

for vasopressin. In most cases the peptide that was found in greater amounts goes on to be the focus of study in that tissue, and thus a role for one but not the other is usually designated. An exception to this is the case of the adrenal gland where early reports indicated greater concentrations of oxytocin than vasopressin, but far more research has gone into elucidating the role of vasopressin in this tissue.

1.6.2.1 Vasculature

Vasopressin immunoreactivity that behaved like synthetic vasopressin in radioimmunoassay and HPLC was extracted from various rat blood vessels and bovine aorta (114). Because of the potential risk for contamination by blood in samples, the researchers took painstaking measures to clarify that this was not the case. Levels of vasopressin extracted from blood vessels were the same in hypophysectomised rats and Brattleboro rats (114). mRNA for vasopressin was identified in both normal (115, 116) and Brattleboro rats (117). The vasopressin V1a receptor is widely expressed in blood vessels and has been shown to be involved in control of blood pressure in situations of haemorrhage, so the role of a locally acting higher concentration of vasopressin is obscure. Vasopressin is a hypertrophic (but not proliferative) agent for cultured smooth muscle cells, and it may be that vasopressin plays a role in the growth of vascular smooth muscle (118, 119).

1.6.2.2 Sympathetic nervous system

There is one report of a vasopressin-like peptide (VLP) in the rat superior cervical and celiac ganglia (120). This immunoreactive substance eluted after the

elution of one void volume, and not with synthetic AVP. Importantly however, when extracted VLP was injected into water loaded rats, that extract was antidiuretic, but had no effect on blood pressure. The authors speculate that VLP is responsible for many of the non-adrenergic responses observed after stimulation of the sympathetic nervous system. Although this novel molecule was shown to have this antidiuretic bioactivity, and have such great potential for neuronal control, no further work seems to have been done to develop these observations.

1.6.2.3 Ovary

The first demonstration of vasopressin immunoreactivity in peripheral tissues was by Wathes *et al* in the human ovary (85, 86), subsequent to observations of larger amounts of oxytocin immunoreactivity (reviewed in (121)). Immunoreactivity was detected at the same elution volume as synthetic vasopressin when subjected to Sephadex G-50 gel filtration. AVP-ir was also shown in the Brattleboro rat corpus luteum (122). Additionally, such immunoreactivity was shown to be 10-fold higher in the adult than that detected in immature rats (122).

mRNA coding for the vasopressin neurophysin has been detected in the ovaries of both Long Evans wild type and Brattleboro rats (123), but gene transcripts analysed by Morley and Ivell could not yield a viable translation product (124). It has been proposed that vasopressin has a role in steroid synthesis regulation in the rat (85), but it seems that oxytocin is the more important NHP peptide in the ovary, and accordingly much more work has been carried out to elucidate mechanisms of expression of that peptide (125). Certainly, no reports of vasopressin receptors in the

ovary could be found when searches were carried out on the literature databases PubMed and Ovid.

1.6.2.4 Uterus

Immunoreactivity specific to vasopressin and oxytocin and their associated neurophysins extracted from rat uterine tissue was characterised. AVP and oxytocin immunoreactivity was shown to have identical mobility with synthetic peptides. The neurophysin immunoreactivity was subjected to a variety of physical separation techniques (gel chromatography, gel electrophoresis, isoelectric focusing) and behaved in the same way as neurophysins extracted from the posterior pituitary gland (126). The neurophysin was also localised to epithelial cells lining the uterus by immunohistochemistry, and was shown to be present in uterine fluid, suggesting secretion (126). In non-pregnant humans, vasopressin levels were higher than those of oxytocin, but not significantly. (127). No reports of the detection of vasopressin mRNA could be found.

The V1a-type receptor was identified in uterine tissue from pregnant rabbits (128) and pregnant humans (129). The mRNA for this receptor has also been identified (130). Levels of the mRNA were shown not to vary throughout gestation (131).

Vasopressin was shown to exert a potent effect on uterine vascular smooth muscle (132), and it is suggested that vasopressin is an important factor in myometrial hyperactivity, uterine ischemia and pain of primary dysmenorrhea as well as regulating blood flow in non-pathological states (133). This is in contrast to oxytocin, which is thought to be involved in the course of labour since an increase in

oxytocin receptors is observed in the uterus just before parturition, and changes in receptor levels are observed at different stages of the menstrual cycle (134). However, activation of the receptors at parturition does not seem to be a function of locally produced oxytocin (135), and the role of the locally produced peptide remains unclear (136, 137).

1.6.2.5 Testis

Vasopressin immunoreactivity has been demonstrated in extracts of testes from the rat (Long Evans and Brattleboro), mouse, dog, and human (138, 139) that was higher than could be accounted for by levels in the blood. The peptide could not be detected however by immunocytochemistry (140). Neurophysin-like immunoreactivity has also been detected in the rat and human testis (138), suggesting that the source of the vasopressin was local synthesis. In addition vasopressin degrading enzyme activity has been detected in extracted testes, which the authors believe may be another necessary criteria for terminating the action of locally produced vasopressin (139).

Accordingly, a faint signal was achieved when Northern blotting analysis was used to probe for the vasopressin mRNA (141). This observation has been successfully repeated by two other groups (142, 143). However, the majority of the transcript that was detected by Lefebvre and Zingg (143) was shown to not contain the vasopressin coding section. This was also demonstrated by PCR of cDNA clones of the transcripts (obtained by differential hybridisation and RT-PCR) (144). Nonetheless, a normal transcript was also detected (in lower levels undetectable by Northern blot analysis), and has been detected in cultured Leydig cells (144), thus

localising the site of production. A promoter element in the rat vasopressin gene specific to transcription occurring in the testis has also been described (145).

With respect to the role vasopressin may play in the testis, the peptide has been shown to inhibit gonadotropin-induced androgen biosynthesis in cultured cells (146), and it has been proposed that vasopressin also acts to stimulate contractile activity of the seminiferous tubules. In keeping with this, vasopressin receptors of the V1a subtype have been demonstrated to be present on Leydig cells and myoid cells of the seminiferous tubules (65, 147, 148). Moreover, Howl and co-workers report that the expression of the receptor on the myoid cells is a developmentally regulated process (they are not apparent in the testis of immature rats), implying that vasopressin has a role in the control of testicular function in the *adult* male rat.

1.6.2.6 Thymus

Vasopressin immunoreactivity was identified in thymus glands of the rat (149) and human (150). Further, immunohistochemistry described in both these studies localised the immunoreactivity to epithelial cells in both the cortex and the medulla regions of the gland. mRNA encoding for vasopressin was identified (151). The V1b receptor has been identified in thymus tissue using RT-PCR (50). It is thought that neuroendocrine peptides expressed by the cells have a role in the negative selection of T-cells that recognise these peptides (152).

1.6.2.7 Pancreas

Immunoreactive vasopressin and oxytocin have been demonstrated in rat and human pancreas (153). Immunochemistry was able to localise both of the peptides to the exocrine portion of the pancreas (154). However, there do not seem to be reports on the presence of vasopressin mRNA in this tissue. A receptor was shown to be present in the pancreas and was pharmacologically characterised as the V1b receptor (155). This characterisation was supported by RT-PCR studies of the pancreas that revealed a positive signal for this receptor (49). Infusion of vasopressin and oxytocin stimulated the release of glucagon and insulin from the pancreas in the rat, dog, and human (156-158), and so it would seem that vasopressin potentially plays a role in modulating the activities of these hormones, perhaps in situations of stress (157).

1.6.2.8 Adrenal glands

By far the largest body of work on an extrahypothalamic source of vasopressin is that which concerns the adrenal glands. Investigation into neurohypophyseal hormones as possible components of the adrenal gland was prompted by an earlier observation that these hormones were present in various areas of the central nervous system (159). Oxytocin had been found in the intermediolateral grey column of the human spinal cord, which is the site of origin of the preganglionic neurones of the sympathetic nervous system (160). The discovery that oxytocin was a component of the ovary prompted another group to look for neurohypophyseal hormones in the testis, and they also examined foetal adrenal glands for some unexplained reason (138).

The first reports of vasopressin (and oxytocin) immunoreactivity in the adrenals were published in 1984. Ang and Jenkins applied extracted rat adrenal glands to a Sephadex G-50 column, and found specific immunoreactivity in the fractions with retention volumes that were the same as for synthetic peptide (159). HPLC was used to separate and identify the neurophysins- they too were detected. Co-localisation of these two molecules suggested that the peptides were being synthesised in the adrenal gland. The same group reported that vasopressin immunoreactivity could be found in the adrenal glands of Brattleboro rats (161). The extracted peptide was shown to be biologically active using a bioassay that measured antidiuretic response in rats. Immunohistochemistry showed the peptide to be present everywhere in the adrenal gland of the Brattleboro rat apart from the zona glomerulosa. No description of detection of the neurophysin is included in this study, which is interesting considering the mutation in the vasopressin gene that causes it to be improperly translated is in the exon that encodes for the neurophysin (162). However, another group showed small amounts of VAG immunoreactivity in some cells of the Brattleboro rat adrenal medulla (163). Nicholson et al showed the presence of both vasopressin and vasopressin-associated neurophysin in the foetal human adrenal (138). This group drew the same conclusion - that co-localisation of both the peptide and the neurophysin in high concentrations argued against their sequestration from the blood.

Other investigators searched for the two peptides in the human and rat foetal adrenal (164). It was theorised that the foetal adrenal might be an alternative source for AVP found in amniotic fluid. Staining was seen in both the definitive cortex and the foetal cortex. In addition, quantitative data supplied for the levels of AVP are

orders of magnitude lower than those quoted by Nicholson (138), being in the range of pg of peptide per g of tissue, as opposed to the 10^2 x pg of peptide per g of tissue. Ravid and co-workers also report very low amounts of immunoreactivity obtained in rat foetal adrenal glands extracts (164). Vasopressin and oxytocin have been described in bovine adult adrenal medulla and cortex, although the proportions of peptide to their respective neurophysin seemed to be variable depending on the tissue examined (165). There was more vasopressin-associated neurophysin in the medulla, despite the fact that equal amounts of vasopressin were observed for medulla and cortex. The ratio of oxytocin to its neurophysin was far closer to molecular equivalency, and they were both present in higher amounts in the medulla than cortex. The biological activities for the extracted vasopressin and oxytocin were also demonstrated in relevant bioassays. The same group examined four different species and found different staining patterns in each (166). Their observation was that staining was generally stronger in the cortex than the medulla, with the zona glomerulosa displaying the strongest staining.

Using RT-PCR, a whole mRNA for the vasopressin gene was demonstrated in the rat adrenal medulla (167). RT-PCR failed to show mRNA for vasopressin in the rat adrenal cortex (168). Prohormone processing enzymes PC1/3 and PC2 have been isolated from bovine (169) and rat (170) adrenal medulla chromaffin cells, indicating that translated preprohormone could be processed in medulla.

It has also been demonstrated that human adrenal medulla chromaffin cells secrete vasopressin under basal conditions (171). Corticotrophin-releasing hormone (CRH) and acetylcholine (ACh) were both shown to stimulate the release of vasopressin from the human adrenal medulla (172). Vasopressin secretion was also

stimulated by ACh in the rat adrenal, but no effect was observed upon infusion of CRH (the peptides are identical in the two species).

Vasopressin receptors have been demonstrated in both the cortex and the medulla of the adrenal gland. Autoradiography demonstrated specific binding of vasopressin in both the zona glomerulosa (173) and zona fasciculata (171) of the human adrenal, with the density of binding two-fold higher in the zona fasciculata (174). Pharmacological characterisation and RT-PCR products identified the presence of only V1a receptors in the cortex (171). Binding sites for vasopressin were demonstrated in the bovine adrenal medulla (175). Taylor and colleagues identified V1-type receptors (176), and RT-PCR of medullary tissue indicated the presence of both V1a and V1b receptors, with V1a receptor present in far greater numbers than the V1b receptor (172). There are no reports of a specific location of the receptors in the medulla, something the authors recommend is addressed as soon as possible (172).

Infusion of human adrenal glands with vasopressin causes aldosterone and cortisol secretion. It was also found that vasopressin had a mitogenic effect on zona glomerulosa cells (177, 178). In rats, it was found that although V1a receptors were present in both the outer zones of the cortex, the receptors in the zona fasciculata were non-functional and unable to stimulate corticosterone secretion (172). Incubation of cultured rat adrenal medulla cells with vasopressin was shown to cause the release of locally stored catecholamines, CRH, and ACTH. (172, 179).

A theory as to the role of locally produced vasopressin is put forward by Guillon and colleagues (172). Stimulation of the splanchnic nerve causes the local release of vasopressin. This causes the release of CRH and ACTH from medulla cells

(a system described as a duplicate of the one seen in the anterior pituitary (180)), cortisol secretion from the zona fasciculata in humans, and cell growth and aldosterone secretion in the zona glomerulosa. Clearly vasopressin is not the only factor effective in the adrenal gland (Nussdorfer examines more than 20 candidate paracrine control molecules (180)) and the redundancy of the system is yet to be established, but several factors are favourable for vasopressin being involved in this way.

A crucial element is the route by which molecules secreted by the medulla are passed to the cortex. There is no recognised blood flow outward from the medulla to the cortex, and so molecules would have to pass to the outer portions of the adrenal by diffusion (an example of paracrine action). This delivery of paracrine controlling elements is aided by the existence of medullary rays. Gallo-Payet and colleagues consistently observed chromaffin cells in all three layers of rat adrenal cortex, sometimes reaching to the capsule surrounding the outer layer (181). Bornstein reported similar observations of chromaffin cells in the cortex of pig, rat, (182) and human adrenal glands (183). Ultrastructural studies in the rat adrenal sometimes revealed chromaffin cells releasing secretory products via exocytosis near adrenocortical cells (184).

Thus, a mechanism for the local delivery of vasopressin to cells, in which the *in vitro* effects of vasopressin have been described, seems to be present in the adrenal gland. Further, Mazzochi describes a theoretical model which rationalises the high concentrations of vasopressin required to exert an effect *in vitro* on adrenal (185). In experiments on the rat adrenal medulla, minimum effective concentration was 10^{-8} M (for future reference, this is equivalent to 10,000 pg AVP/ml) (179), whereas blood

plasma AVP concentrations are quoted as rarely exceeding 5×10^{-11} M (or 50 pg/ml, attributed to (186) in (179)). For a molecule to be genuinely considered to be exerting paracrine control, he stipulates that it must be possible for the adrenal content of the molecule to generate a local concentration of the molecule equal to the minimum effective concentration *in vitro*. He states that the specific gravity of adrenal gland is 1.039, and the interstitial space in the adrenal gland less than 2-3% of the total volume. Nussdorfer used this model to make some rough estimations. Adrenal glands containing molecules with a content of 100 femtomoles/g will generate a concentration of 10^{-9} M if 30% of their content is released from cells. He calculates that AVP concentrations as high as 10^{-7} M could be generated in the rat adrenal gland, and 10^{-6} M in the human (180).

Adrenal glands represent a well-examined model of a potential paracrine role for vasopressin, and should be used as such when considering novel candidate sites for vasopressin synthesis and local action.

1.7 Vasopressin in foetal skeletal muscle

It has been proposed that foetal skeletal muscle be added to the list of tissues that synthesise and locally use biologically active arginine vasopressin. In 1992, a report was published in *Early Human Development*, demonstrating high concentrations (between 10 and 0.04 **ng** of AVP per g tissue) of apparently specific vasopressin immunoreactivity in human foetal skeletal muscle (187). The authors demonstrated this immunoreactivity in a variety of ways. A graphed relationship between the immunoreactivity from the muscle and gestation age showed that as the age of the foetus increased, the immunoreactivity per unit tissue weight in the muscle

decreased. After high performance liquid chromatography (HPLC), the immunoreactivity was found to dilute in parallel with an extract of pituitary gland and synthetic AVP treated in a similar manner. Further techniques were used to locate the immunoreactivity within a molecular weight range. Molecular weight cut off (MWCO) filters were used to show that the immunoreactivity had a molecular weight of less than 3kDa. Examples presented showed there was no loss of immunoreactivity when the extract of foetal muscle was filtered through one or two MWCO filters and then further extracted using HPLC. The mobility of the immunoreactivity was shown to be the same as that of synthetic AVP, as radioactive peaks due to tritiated AVP and immunoreactive peaks from the assay of the extract had the same retention time. Immunoreactivity was also demonstrated in other AVP assays using three different antibodies raised against a vasopressin antigen.

The observations of Smith and colleagues fit well with data gathered later in other laboratories concerning the action of vasopressin on skeletal muscle tissue. Initial studies designed to investigate the possible effect vasopressin has in the carbohydrate metabolism of skeletal muscle employed chick embryo myoblasts. The transient effect of vasopressin on glucose 1,6 – bisphosphate (a strong regulator of several enzymes involved in carbohydrate metabolism) was examined (188). In pre- and post-fusion myoblasts, vasopressin was observed to lower levels of glucose 1,6-bisphosphate. The authors drew no conclusions as to whether these changes were due to a decrease in synthesis of the bisphosphate from glucose 1-phosphate, or to an increase in the degradation of the molecule itself. However they reasonably concluded that a peptide affecting the levels of a compound involved in the carbohydrate metabolism pathway must be influencing conditions or enzymes within

that pathway. Vasopressin has been shown to produce a glycogenolytic effect on the liver in the rat and mouse. In the perfused rat liver and in hepatocyte preparations, vasopressin has been reported to cause both the release of glucose and the stimulation of glycogen phosphorylase activity (189). This was shown to operate through a cAMP-independent pathway, implying the involvement of a V₁-type receptor in the initiation of the glycogenolytic response to vasopressin (190).

Wakelam and colleagues then went on to study the transient response to vasopressin in the L6 rat myoblast cell line (191). It was shown that vasopressin stimulated inositol phospholipid breakdown, thereby linking the two strands of observations in liver and skeletal muscle. No effect on intracellular cAMP concentrations was seen, establishing both the lack of functional V₂-type receptors and the presence of V₁-type receptors, and suggesting further that any effect vasopressin has on skeletal muscle is mediated via this receptor. On the basis of these findings Adamo and colleagues were prompted to further study the signal transduction events and the long term effects of vasopressin on the L6 cell line. L6 cells are immortalised myoblast cells that retain the potential to differentiate into the mature skeletal muscle phenotype (192). Skeletal muscle fibres are multinucleated non-mitotic cells, formed from the fusion of progenitor mononucleated myoblasts (193). When vasopressin was introduced into a culture of L6 myoblasts, concentration dependent inositol phosphate production was observed (194). In addition, a biphasic increase of intracellular calcium occurred. The onset of these effects were seen within 2-3 seconds of the introduction of AVP. Vasopressin was also able to alter the internal pH of cells. A hypothesis was extended that AVP was acting as a proliferative factor, and the effect of long-term exposure to AVP was then

examined (195). Incubation of cells with AVP in the culture medium resulted in an increase in fusion of myoblasts. L6 cells were seen to fuse without the addition of AVP but vasopressin increased the rate of this fusion two-fold, and fused cells were observed to contain higher a number of nuclei. It was later shown that L6 cells could be held in the non-functional state almost indefinitely with manipulation of the culture medium (196). The signal transduction pathways activated by vasopressin in L6 cells was further studied and it was shown that phospholipases C and D were activated (197, 198). Vasotocin and oxytocin exerted the same effects as vasopressin on the differentiation of the cells at the same concentration (0.1 μ M), but only the V1a receptor was identified through use of selective antagonists of the different vasopressin receptors.

1.7.1 Foetal development and role of vasopressin

Describing the ontogeny and roles of the neurohypophyseal hormones in the foetus is hampered by the availability of samples for study and the gestation age of the foetuses used for study. Much of the data available on the function of the hormones in the foetus are gathered from the chronically catheterised sheep foetus. These studies are usually carried out late in the third trimester. Human samples available for study are not fresh, being gathered from legal therapeutic terminations with inevitable delay between delivery and sampling, and are not comparable in terms of stage of gestation, even if the differences in the development of sheep and humans can be overcome. In this section, therefore, it should be borne in mind that referring to the foetus is usually a reference to observations made on the third trimester foetus.

The anatomical components of the hypothalamo-neurohypophyseal system are established and developing by the 7th week of gestation in the human (199). Reports vary, but it seems that the demonstration of vasopressor activity is not possible before week 10 (21). Vasopressin has been detected in human foetal posterior pituitaries as early as the 11th week of gestation (200). In early gestation foetuses a high vasopressin to oxytocin ratio is observed. This is due to a time difference between the appearance of oxytocin and vasopressin in the posterior pituitary - vasopressin is detectable 3-4 weeks before oxytocin (201). In the adult human posterior pituitary gland, vasopressin, oxytocin and their respective neurophysins are present in roughly equal proportions (202), but this ratio is not seen until at least the neonatal period (21). However, the ratio of the individual neurophysins remains relatively constant despite a thousand-fold increase in tissue stores in the ensuing 3-4 months. Thus, the changes in the ratio of vasopressin to oxytocin are not due to relative changes in rates of *de novo* synthesis, and instead have been explained by the later maturation of processing machinery in the oxytocin producing cells (201).

Vasopressin seems to be able to play a role in adaptation to intrauterine stress. Basal and raised circulating levels of vasopressin are due to foetal release because vasopressin and oxytocin do not cross either ovine or human placenta (203). Although basal levels of either hormone have not been established in humans, vasopressin has been measured in ovine foetal blood at mid-pregnancy (204). Basal maternal levels in the sheep are similar to non-pregnant ewes and human adults, as are foetal vasopressin levels in sheep.

The internal environment of the foetus is ostensibly controlled by the mother. However, vasopressin is capable of affecting conditions in foetal physiology. Elevated levels of vasopressin affect the foetal renal, lung and cardiovascular systems. By mid-term, the foetus is capable of increasing circulating levels of vasopressin in response to osmotic stimuli (205), hypoxia as a result of maternal hypoxia (206), haemorrhage (207), and dehydration of maternal origin (204). The foetal response to hypoxia is proportional to the degree and duration of the episode (208). Foetal lung fluid production is diminished by vasopressin infusion (209). In the short term, therefore, vasopressin can diminish water loss through the foetal lung. Water loss can also be controlled by the foetal kidney, but the kidney is not fully functional. The response to stimuli like these matures with increasing gestation age so that only by birth are secretory responses mediated by osmolar and volume receptors similar to those in the adult. This maturation is demonstrated by the antidiuretic capabilities of the foetus. Under basal conditions, foetal urine osmolality is significantly lower than plasma osmolality. Even during infusion of exogenous vasopressin, maximal foetal urine osmolality rarely exceeds plasma osmolality.

Cardiovascular responses to vasopressin are also seen. There is a redistribution of the cardiac output to spare the placental, myocardial and cerebral circulation (210).

Increased vasopressin secretion elevates foetal blood pressure and leads to bradycardia. Parturition is also associated with increased ovine foetal vasopressin at a time when there is no concomitant maternal secretion. This is undoubtedly a response to the mild hypoxia associated with labour, because in caesarean section deliveries cord blood vasopressin levels are only minimally elevated at birth.

1.8 Proposal of a model for the action of vasopressin in foetal human skeletal muscle

It would seem obvious that a circulating concentration of vasopressin necessary to mimic the effects on skeletal muscle observed *in vitro* could have profound effects on the normal physiology of the foetus, and so the remaining option is the local synthesis of smaller amounts of vasopressin that would generate the required concentrations to exert an effect with a paracrine or autocrine action. A model of vasopressin action on foetal skeletal muscle is therefore proposed, whereby vasopressin is present in the high levels reported in early gestation in order to promote the fusion of myoblasts, generating multinucleated myofibrils, and thus promote cell growth and maturation to form functional units of muscle. As gestation progresses, the requirement for vasopressin-induced fusion is lessened, as the ratio of myoblasts to mature muscle cells progressively lowers, and so production of vasopressin is lowered. This could happen in one of two ways. If the muscle cells themselves produce the vasopressin (an autocrine action), then the simple effect of the lowering of the number of cells in the muscle will result in this lowering of concentration. If, however, it is an associated cell type that produces the vasopressin (a paracrine action), then the rate of synthesis and release of vasopressin would need to be down-regulated in some manner.

1:9 Conclusion

The following chapters describe the experiments carried out to investigate the possibility that vasopressin is found at physiologically significant levels in foetal

human skeletal muscle. Employing the criteria set out in Section 1.6 and using the example of the adrenals glands, it is proposed that the synthesis of vasopressin in skeletal foetal muscle should be examined in a stepwise manner, in order to extrapolate the observations in the rat myoblast L6 cell line to those of human foetal muscle.

Initially, confirmation of the immunoreactivity in human foetal muscle should be carried out using radioimmunoassay. The localisation of the source of the immunoreactivity should be investigated, using immunohistochemistry or *in situ* histochemistry. Generally, it should be established if it is possible to isolate a viable vasopressin mRNA from the muscle tissue, using either RT-PCR or Northern blotting. The presence of enzymes involved in the maturation of translated gene should be demonstrated to show that the preprohormone can be processed to give an active final product.

A vasopressin receptor should also be localised in order to establish that the vasopressin signal can be transduced. Presumably, content of the receptor will also diminish with time, but as the action of vasopressin on mature skeletal muscle has been described (188), this is not certain. The concentration of the receptor on the surface of the myoblast could be found using membrane preparations, and a mRNA for the receptor should be sought using either RT-PCR, Northern blotting, or *in situ* hybridisation. The messenger cascades stimulated by vasopressin binding should also be investigated on primary cultures of human myoblast cells.

The following chapters describe the experiments carried out in an effort to reproduce the observations of Smith *et al* that high levels of vasopressin were found in the skeletal muscle of human foetuses (187). Additionally, the presence of the

V1a-vasopressin receptor was examined using Western blotting and immunohistochemistry.



Chapter 2

Materials and Experimental Procedures

2.1 Samples used in studies

2.1.1 Samples used in experiments for the extraction, gel filtration, and radioimmunoassay of vasopressin

Adult rabbit skeletal muscle was taken from an adult female New Zealand White rabbit, kindly donated by the Faculty of Medicine Animal Facility (FMAF, Faculty of Medicine, University of Edinburgh, UK). The rabbit was sacrificed by lethal injection of anaesthetic. The quadriceps muscle was immediately dissected out, divided into portions, snap frozen, [MFL1]and stored at -70°C until used.

Adult rat adrenal glands were taken from adult female Sprague Dawley rats, kindly donated by the FMAF. The rats were sacrificed by suffocation using CO_2 gas, and after transportation, the adrenal glands were dissected out, weighed and snap frozen.

Foetal human muscle, liver, pituitary, and adrenal glands representing different stages of mid-trimester development were taken from therapeutic terminations carried out at the Simpson's Memorial Hospital, Edinburgh, UK. Tissues were obtained as quickly as possible after the procedure. Ethical consent was sought and received from all donors at the time of the procedure, in accordance with a study protocol approved by the Paediatrics & Reproductive Medicine Research Ethics Sub-Committee for the Lothian Health Board (Protocol reference

1702/94/6/6). All fetuses were anonymously donated and processed in accordance with guidelines set out in the Polkinghorne Report (211). Only those fetuses without obvious external malformation or pathology were released for study.

An estimated gestation age was supplied with each fetus, but it is not known how definitive this estimate was. Gestation ages were generally assigned as being a whole or half a week, for example, 15 weeks, 15+ weeks, 16 weeks. This gave rise to concerns regarding the accuracy of gestation age and any attempts to correlate this age with the actual development of the fetus. Accordingly, the fetus was weighed, and the foot length measured using the directions of Hern (212). The foot was placed flat on a ruler, and the foot length measured from the back of the heel to the tip of the longest toe (usually the second toe). Both feet were measured, and the mean noted as the foot length. Foot length has been shown to be a reliable indicator of gestation age (first defined by Streeter in 1920(213)), and this parameter can be used as a marker of foetal development (212, 214, 215).

Foetal human quadriceps muscle, adrenal glands, and the pituitary gland as a whole (the middle cranial fossa was cut away from the base of the skull) were dissected from the fetus, snap frozen and stored at -70°C .

2.1.2 Samples used in immunoblotting experiments

Samples of adult rabbit and foetal human quadriceps muscle (obtained as described above) and foetal human liver were defrosted, weighed, and then refrozen. These samples were then freeze dried and stored at -20°C prior to transportation at room temperature.

5×10^7 MCF-7 cells for a positive control preparation, and an adult human liver positive control sample were kindly donated by Dr William North (Department of Physiology, Dartmouth Medical School, Dartmouth College, Lebanon, NH, USA).

2.1.3 Samples used in immunohistochemistry experiments

Foetal human skeletal muscle, pituitary and adrenal glands (dissected from foetuses as described above) were immediately fixed in 4% phosphate buffered formalin solution. Tissues were subsequently processed by gradual removal of the fixative and embedded in paraffin wax by staff in the Pathology Department at the Royal Hospital for Sick Children, Edinburgh, UK.

Adrenal glands were taken from adult female Long-Evans rats immediately after sacrifice by decapitation. They were fixed in 10% phosphate buffered formalin. Fixative was removed and the tissues were embedded in paraffin wax by staff in the Histotechnology Section of the Department of Anatomical Pathology at the Dartmouth Hitchcock Medical Center (DHMC), Lebanon, NH, USA.

Adult human hypothalamus and adrenal gland tissue were cut from control blocks of normal hypothalamus and adrenal gland tissue kindly provided by the Department of Anatomical Pathology at the Dartmouth Hitchcock Medical Center. In order to protect patient confidentiality, information as to the source of the tissues was not made available.

Slides were stained using hemotoxylin and eosin using facilities in the Histotechnology Section of the Department of Anatomical Pathology at DHMC.

2.2 Materials

2.2.1 Materials used in the extraction and radioimmunoassay of vasopressin

All solutions were prepared using double distilled water.

Tissues were thoroughly homogenised in 1M acetic acid (from glacial acetic acid, HPLC grade, Fisher Scientific UK Ltd.) using a Kinematica Polytron homogeniser (Brinkmann Instruments Inc., Westbury, NY, USA). Homogenisations were carried out in 50ml conical tubes (Sarstedt Ltd., Beaumont Leys, Leicestershire, UK).

All assays and binding experiments were carried out in LP2 tubes (Sarstedt Ltd.). Radioimmunoassays were counted using a Cobra II Autogamma counter (Packard Bioscience Ltd., Berkshire, UK).

2.2.1.1 Buffers and solutions

Assay buffer was based on 0.05M Tris-hydrochloric acid (Tris-HCl) buffer. Tris (SigmaUltra grade, Sigma-Aldrich Company Ltd., Poole, Dorset, UK) was weighed out and the pH adjusted to 7.2 using 5M (5N) hydrochloric acid (Standard Volumetric Solution, Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK). 0.3% (w/v) bovine serum albumin (BSA, RIA grade fraction V, Sigma-Aldrich Company Ltd.) was added. 0.1% (v/v) Triton X-100 and 0.05% (w/v) sodium azide (both SigmaUltra grade, Sigma-Aldrich Company Ltd.) were also added. The buffer was prepared in batches of 1 litre and stored at 4°C. Primary antibodies, AVP standard, and radio-labelled AVP and samples were all diluted to appropriate concentrations in this buffer.

Adjustment of the pH of extracted samples containing 1M acetic acid was carried out using 2M Tris (SigmaUltra grade, Sigma-Aldrich Company Ltd.). pH was checked using a pH meter (Accumet pH Meter 915, Fisher Scientific UK Ltd.). Tris was added until the pH of a sample reached 7.2.

Bound and unbound portions of the assay were separated by the total precipitation of the primary antibody using a second antibody directed against the species in which the primary antibody was raised. Precipitation of an antibody-antigen complex only occurs at high concentrations of the antibody and so is not seen in other parts of the assay. Precipitation is brought about by adding normal serum from the primary antibody species to provide the required high concentration of antibody-antigen complex. Precipitation is further enhanced by the use of polyethylene glycol (PEG) which absorbs excess water (216). Second antibody solution sufficient for 200 tubes was made using 24ml of assay buffer, 24ml of an 8% (w/v) PEG 8000 solution (SigmaUltra grade, Sigma-Aldrich Company Ltd.), 2ml of neat donkey anti-rabbit serum for assays using *tg1* antiserum (or anti-sheep for assays using the *s278* antiserum) and 200µl normal rabbit serum for the *tg1* assay (or sheep serum for the *s278* assay). These sera were all supplied specifically for the development of, and use in, radioimmunoassays by the Scottish Antibody Production Unit (SAPU, Lanarkshire, UK). Precipitated bound tracer was sedimented by centrifugation using a Jouan CR 422 centrifuge (Jouan Ltd., Ilkeston, Derbyshire, UK).

Storage buffer was used to dilute and store AVP standard and ¹²⁵I-labelled AVP and was made when required. This buffer consisted of 0.2% (v/v) acetic acid

(Fisher Scientific UK Ltd.) and 0.2% (w/v) BSA (Sigma-Aldrich Company Ltd.) in distilled water.

2.2.1.2 Radio-labelled peptides, peptide standards and analogues

Iodinated (^{125}I -labelled) AVP (Amersham Pharmacia Biotech UK Ltd.) was used as the tracer in all radioimmunoassays. The half life of ^{125}I is approximately sixty days, and tracer was replaced within this period. No change in the stability of the tracer was apparent in this time. Each consignment was divided into aliquots, each of which provided sufficient radioactivity for approximately 1000 assay tubes (5000 counts per minute (cpm) per tube) in 50 μl . Unused tracer was stored at 4°C and used within four days. Disposal of tracer was carried out according to local regulations.

Peptide labelled with a radioactive isotope was also used to evaluate extraction efficiency of the procedure and to standardise elution of peptide in gel filtration chromatography experiments. AVP labelled with tritium (^3H -AVP) was purchased in 50 μCi batches (Amersham Pharmacia Biotech UK Ltd.). Samples containing radioactive peptide were counted in quadruplicate. Aliquots were mixed with Picofluor 40 liquid scintillation cocktail (Packard Bioscience Ltd.) and disintegrations per minute (dpm) were counted for a two minute period using a Tricarb 1900 CA liquid scintillation analyser (Packard Bioscience Ltd.).

Synthetic AVP was used for the generation of standard curves in all experiments, and in experiments where AVP was added as a supplement (BACHEM (UK) Ltd., St. Helens, Merseyside, UK). Aliquots of 100ng/ml concentration were

prepared in storage buffer and stored at -20°C. A new aliquot was used every time a standard curve was set up.

The 1st International Standard for Vasopressin for Bioassay (National Institute for Biological Standards and Control, London, UK) was used in the radioimmunoassay as the standard reference material, and for quality control samples. An ampoule was broken and dissolved using storage buffer to give aliquots of 1 µg/ml, which were stored at -70°C. These aliquots were diluted in assay buffer to make three different concentrations of reference standard which were stored at -70°C and used in every assay. Two different sets of reference pools were created; one for the *tg1* and one for the *s278* assay. The concentrations were chosen so as to provide a high (20% binding) medium (40% binding) and low (70% binding) dose when assayed as a sample in an assay. Quality control pools were changed every three months.

The specificities of the *tg1* and *s278* antisera were tested using lysine vasopressin, oxytocin and vasotocin (BACHEM (UK) Ltd.), structurally similar peptides that are also found in mammals.

2.2.1.3 Antibodies used as binders in the radioimmunoassay

tg1 is a polyclonal antiserum. It was raised in rabbits using bovine thyroglobulin conjugated arginine vasopressin (AVP). Use of this antibody has been described in several publications (187, 217-219).

s278 is a polyclonal antiserum raised in a sheep. It was raised at the Scottish Antibody Production Unit (SAPU, Lanarkshire, UK) facility using bovine thyroglobulin conjugated AVP. Information concerning the number and timing of the

immunisation injections was not available. Serum was pooled from several bleeds, and stored at -20°C until used. Use and partial characterisation of the s278 antiserum has been described before (187, 217, 218).

2.2.1.4 Materials used in solid phase extraction

Isolute End Capped octylsilyl-silica (C8 silica) cartridges were used for the solid phase extraction of vasopressin from extracted tissues (International Sorbent Technology, Hengoed, Mid-Glamorgan, UK). Each cartridge contains 500mg of adsorbent, with a 10ml reservoir for addition of solutions and samples. Solutions and samples were drawn through the adsorbent using a Varian Vac-Elut 10 vacuum manifold (Varian Inc., Walton-on-Thames, Surrey, UK). The vacuum pressure was changed at the different steps to cause different flow rates, but pressures were not quantified. Eluates of interest were dried under vacuum using a Gryovap centrifuge (Philip Harris Scientific, Ashby de la Zouch, Leicestershire, UK).

Prior to use, the cartridge adsorbent was stripped with methanol (Certified HPLC grade, Fisher Scientific UK Ltd.) then washed with an 80% aqueous acetonitrile (ACN, Certified HPLC grade, Fisher Scientific UK Ltd.) solution containing 0.2% trifluoroacetic acid (v/v) (TFA, HPLC grade, Fisher Scientific UK Ltd.). ACN was washed out using aqueous 0.2% TFA solution. Samples were loaded to dryness, and the retained sample was washed (to remove weakly bound impurities) with aqueous 0.2% TFA solution. Samples were eluted with 80% aqueous ACN containing 0.2%TFA. The cartridges were regenerated by washes with methanol, followed by 80% aqueous ACN solution containing 0.2%TFA, and finally

0.2% aqueous TFA solution. The cartridges could be used 3 times before being discarded, with no loss of extraction efficiency (data not shown).

2.2.2 Additional Materials used in gel filtration experiments

Gel filtration chromatography was carried out using Sephadex G-25 gel (Fine grade, Sigma-Aldrich Company Ltd.). Weighed quantities (47g) of gel were swollen overnight in 250ml of 0.05M Tris-HCl (pH 7.2) buffer at 4°C. Swollen resin was packed into a 70cm (length) x 16mm (internal diameter) chromatography column ('C' type, Amersham Pharmacia Biotech UK Ltd.) under gravity flow. All samples were eluted in 0.05M Tris-HCl (pH 7.2) buffer and experiments were carried out at 4°C. The void (or exclusion) volumes were established using Blue Dextran, a coloured marker with an average molecular weight of 2×10^6 Da (Sigma-Aldrich Company Ltd.), and was found to be approximately 44ml. Fractions were collected using a Gilson 201 fraction collector (Anachem Ltd., Luton, Bedfordshire, UK).

Fractions from gel filtration experiments were sometimes pooled and concentrated using solid phase extraction. The resulting samples were dissolved in assay buffer and centrifuge filtered with a molecular weight cut off device that had a cut off weight of 10kDa (Microcon YM-10, Millipore (U.K.) Limited, Watford, Hertfordshire, UK).

2.2.3 Additional materials used in immunoblotting experiments

2.2.3.1 Lysis buffer and other solutions and buffers used in electrophoresis, transfer, and Western blot analysis

Weighed portions of freeze dried samples were extracted for protein in one of two ways. Some samples were extracted using TRIzol reagent (Life Technologies, Rockville, MD, USA), a monophasic solution of phenol and guanidine isothiocyanate that can be used to isolate total RNA, DNA, and protein from the same sample. Other samples were extracted for protein in lysis buffer. The lysis buffer was 0.01M ammonium chloride solution buffered with 0.05M Tris-HCl (pH7.5), and also contained proteolytic enzyme inhibitors 0.2U/ml aprotinin, and 1mM phenylmethylsulfonylfluoride (PMSF, both Sigma-Aldrich Company Ltd.) (220). This buffer has been used in the lysis of cultured cells for Western blot analysis of vasopressin receptors (221).

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS - PAGE) was carried out using standard electrophoresis running buffer containing Tris, glycine and SDS (purchased as a premixed 10x buffer, Bio-Rad Laboratories, Hercules, CA, USA) (222).

Proteins were transferred from gel to membrane using a standard transfer buffer containing 15% methanol (Certified HPLC grade, Fisher Scientific, Pittsburgh, PA, USA), Tris and glycine (pH 8.3, made according to instructions in (222)).

Between additions of antibodies, membranes were washed in standard phosphate buffered saline solution (PBS, pH 7.3, made according to instructions in (222)) containing 0.1% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20,

Electrophoresis grade, Sigma-Aldrich Company Ltd.). Antibodies were also diluted and incubated with the membranes in this buffer with 5% (w/v) BSA (RIA grade fraction V, Sigma-Aldrich Company Ltd.) added.

2.2.3.2 Antibodies used in immunoblotting analysis

Antibody used in the detection of the V1a receptor subtype (*vivian*) was generated using a peptide conjugated to bovine thyroglobulin. The peptide used was a sequence unique to the human form of the receptor, and comprised part of the second putative extracellular loop and a portion of the transmembrane region 5 of the receptor structure (221). The antibody had previously been used in a radioimmunoassay for the receptor using the radio-iodinated peptide, and in Western blot analysis of vasopressin receptors in cell lines (221). The rabbit antiserum had previously been fractionated (using Immunopure Immobilized Protein-A, Pierce, described in (221), and the IgG2b fraction was used at a concentration of 10µg/ml.

Foetal muscle samples were normalised for content of relevant protein using a mouse monoclonal antibody directed against human β -dystroglycan (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK). This protein is a member of a group of proteins known as dystrophin associated glycoproteins (DAGs), and is thought to be involved in the attachment of dystrophin to muscle membranes (223). It has been used in the study of the developmental expression of membrane cytoskeletal proteins in human skeletal muscle (224).

2.2.3.3 General materials used in immunoblotting experiments

Samples extracted using TRIzol reagent were homogenised using a Kinematica Polytron homogeniser (Brinkmann Instruments Inc.).

Samples were homogenised in lysis buffer using a sonicator (Model W-380, Heat Systems - Ultrasonics, Inc., Farmingdale, NY, USA). Homogenates were spun at 12,000g for 10 minutes using an Eppendorf 5415C centrifuge (Eppendorf Scientific, Inc., Westbury, N.Y, USA). Supernatants were decanted and stored at -20°C until used.

Protein content of the samples was estimated using a protein assay kit (BCA-200 Protein Assay Kit, Pierce, Rockford, IL, USA). Prior to electrophoresis, samples were mixed with 2x sample buffer containing Tris, glycerol, SDS, and bromophenol blue (Laemmli Sample Buffer, Bio-Rad Laboratories, Hercules, CA, USA) with, or without, 0.05M dithiothreitol (DTT, electrophoresis grade, Sigma-Aldrich Company Ltd.). Pre-stained protein standard markers were added to a separate well for the purpose of estimating molecular weights of identified proteins (Kaleidoscope Pre-stained Standards, Bio-Rad Laboratories, Hercules, CA, USA).

SDS-PAGE and electro-blot transfer was carried out using Biorad Mini-Protean 3 cell and mini-Trans Blot apparatus (Model 1000/500 powerpack, Bio-Rad Laboratories, Hercules, CA, USA). 12% polyacrylamide Tris-SDS gels were prepared according to the method of Laemmli (222). Separated proteins were transferred to PVDF membrane (Immobilon-P transfer membrane, Millipore Corporation, Bedford, MA, USA). Membranes were dried using a gel dryer (Model 583, Bio-Rad Laboratories, Hercules, CA, USA).

Binding of *vivian* to target antigen was detected using a horse radish peroxidase (HRP) conjugated goat anti-rabbit IgG (Premium Quality, Human serum adsorbed, Gibco BRL Life Technologies, Rockville, MD, USA). Binding of the anti-beta-dystroglycan antibody was detected using a horse radish peroxidase (HRP) conjugated goat anti-mouse IgG (ImmunoPure Goat Anti-Mouse IgG, Pierce).

Immunoreactive proteins were visualised by incubating the membrane in chemiluminescent substrate (Lumi-Light Western Blotting Substrate, Roche Diagnostics Corporation, Indianapolis, IN, USA), and photographic film (Super RX X-ray film, Fuji Film USA) was exposed to the membrane in a darkroom. Films were developed using a Kodak RP –Omat processor (Model M7B, Eastman Kodak Co., Rochester, NY, USA).

2.2.4 Additional materials used in immunohistochemistry experiments

2.2.4.1 Tissue sectioning

Paraffin embedded tissues were cut into 5µm sections using a microtome (RS4800 model, Surgipath Medical Industries Inc., Richmond, IL, USA), laid out in a water bath set at 37°C (tissue floatation bath, Surgipath Medical Industries Inc.), and transferred to microscope slides that had been previously coated with a tissue section adhesive according to manufacturers instructions (Biobond, BBIInternational, Cardiff, UK). The sections were air dried at room temperature for at least 12 hours before use.

2.2.4.2 Immunohistochemistry buffer and other solutions

Immunohistochemistry buffer was a 0.05M sodium phosphate buffered (pH7.5, Sigma-Aldrich Company Ltd.) 0.9% (w/v) saline (sodium chloride, Sigma-Aldrich Company Ltd.) solution. The buffer also contained 0.25% (w/v) Triton X-100 (Sigma-Aldrich Company Ltd.). The buffer was used as a wash between applications of reagents and used to dilute antibodies and normal sera.

Paraffin was removed from tissue sections using xylene (Histological grade, Fisher Scientific) and the tissues rehydrated using decreasing concentrations of alcohol (diluted from absolute ethyl alcohol, Aaper Alcohol, Shelbyville, Kentucky, USA). Endogenous peroxidase activity, responsible for background staining where enzymes were active, was blocked using 0.3% hydrogen peroxide (from 30% stock, certified ACS grade, Fisher Scientific) in methanol (Fisher Scientific).

Antigen retrieval was carried out either by heating the slides in a Samsung MT4600 microwave oven using Antigen Unmasking Solution (Vector Laboratories Inc., Burlingame, California, USA), or by incubation with trypsin (DAKO Corporation, Carpinteria, CA, USA). Trypsin was removed by washing with 95% ethanol (Aaper Alcohol, Shelbyville, Kentucky, USA)

Normal serums were used as negative controls. Binding of the primary antibody was demonstrated using Universal Biotinylated Antibody (a mixture of biotinylated anti-rabbit and anti-mouse antibodies, Vector Laboratories Inc.). Avidin Biotin Complex (ABC kit) was applied, and colour was developed using a 3'3'-diaminobenzidine (DAB) kit. Slides were counter stained with hemotoxylin solution. (All the above were purchased from Vector Laboratories Inc.)

Immunohistochemistry experiments were carried out using coverplates (Shandon Inc., Pittsburgh, Pennsylvania, USA). These are moulded covers that create a uniform channel over the tissue section. Use of these coverplates allows lower volumes of reagents to be used and provide control over addition of set volumes of solutions to the slide surface. Additionally, the coverplates are designed to keep a constant layer of solution over the tissue section, preventing the slide from drying out at any stage in the procedure.

2.2.4.3 Antibodies used in immunohistochemistry experiments

Vimentin was detected using a mouse monoclonal antibody (BioGenex Laboratories Inc., San Ramon, CA, USA). Vimentin is an intermediate filament that has been shown to be present in foetal skeletal muscle until 36 weeks gestation (225). It has also been used as a marker for certain tumour types (226).

Vasopressin was detected using a rabbit polyclonal antiserum (*gonzo*). The antibody was generated to vasopressin coupled to bovine thyroglobulin and has a cross reactivity of less than 0.01% with oxytocin (227). It has been used in the immunohistochemical investigation of vasopressin expression in gastrointestinal cells of rats (227). The antibody was used as a dilution of the neat serum without purification.

vivian, an anti-vasopressin V1a receptor antiserum (described in Section 2.2.3.2) was also used.

2.3 Experimental procedures

Statistical analysis was carried out using InStat 2.03 (Graph Pad Software Inc., SanDiego, CA USA), a statistical package for the Macintosh. Lines of linear regression and curve fits on graphs were imposed by algorithms resident in the software of Microsoft Excel 98 (Microsoft Corporation).

2.3.1 Homogenisation and Extraction

Tissues were thoroughly homogenised in hot molar acetic acid using a Polytron homogeniser. Homogenisations were carried out in 50ml conical tubes. Samples were then boiled for ten minutes and homogenised again. Homogenates were centrifuged at 12,000g for 45 minutes at room temperature.

2.3.2 Vasopressin Radioimmunoassay

2.3.2.1 Basic protocol

Both the *tg1* and *s278* assays were set up in the same manner. Four different types of tubes were set up: Total Counts tubes, Non-Specific Binding tubes, B₀ tubes, and Sample or Standard tubes. All measurements were carried out in triplicate. The assay is summarised in Figure 2.1. Standard curves were made appropriate to the antiserum used in the assay. *tg1* assay standard curves were set up over a range of 100-1pg/ml (1-100pM), and *s278* assay standard curves covered a range of 7-2000pg/ml (7-2000pM).

On day 1, nothing was added to Total Counts tubes. 100µl of assay buffer was added to Non-Specific Binding tubes. Primary antibody was added to B₀ tubes in 50µl, and together with 50µl of assay buffer. Sample tubes had 50µl of sample (or

AVP standard) and 50µl of primary antibody added to them. The tubes were mixed by vortexing and incubated overnight at 4°C.

On day 2, ¹²⁵I-AVP (5000 counts per tube) was added to all tubes. All tubes were mixed by vortexing, then incubated overnight at 4°C.

On day 3, 250µl of second antibody/PEG mixture was added to all tubes except the Total Counts tubes. Tubes in which second antibody/PEG mixture had been added were incubated for 1 hour at 4°C, and then centrifuged for 20 minutes at 2,500g at 4°C. The supernatant (containing the unbound fraction) were aspirated and discarded from each tube, leaving only the pellet (containing the bound fraction) in the tube. All tubes were counted for 5 minutes. Assays were counted using the Cobra II Autogamma counter. The built-in computer constructed standard curves using a 4-parameter logistic fit, and values for the concentration of vasopressin in samples were extrapolated within the same program.

2.3.3 Solid phase extraction

The extraction protocol is similar to that preferred by Van de Heijning and colleagues (228). Prior to use, the cartridge was stripped with a reservoir volume (10ml) of 100% methanol, then washed with 4.5ml of an 80% aqueous ACN containing 0.2% aqueous TFA solution. This wash 'activated' the cartridge for sample loading. Two 10ml volumes of 0.2% aqueous TFA solution were washed through the resin to remove ACN. Over these first steps it was found to be imperative that the adsorbent did not dry out.

Samples that did not contain acid were supplemented with 0.2% TFA solution. Samples were then added to the cartridge reservoir, and vacuum pressure

	Total counts tube	Non-specific binding tube	B ₀ tube	Sample/ standard tube
<u>DAY 1</u>				
Sample/standard	-	-	-	Add 50µl
Primary antibody	-	-	Add 50µl	Add 50µl
Assay buffer	-	Add 100µl	Add 50µl	-

Incubate overnight at 4°C

<u>DAY 2</u>				
¹²⁵ I-AVP (5000 counts per tube)	Add 50µl	Add 50µl	Add 50µl	Add 50µl

Incubate overnight at 4°C

<u>DAY 3</u>				
Second antibody /PEG mixture	-	Add 250µl	Add 250µl	Add 250µl

Incubate for 1 hour at 4°C

Centrifuge tubes (except total counts) at 2500g for 20 minutes at 4°C

Aspirate and discard unbound portion (supernatant).

Count all tubes for 5 minutes

FIGURE 2.1 Basic protocol for vasopressin radioimmunoassay

was applied so that each sample was loaded at a rate of less than 1ml every two minutes, to ensure sufficient exposure of the sample to the adsorbent. Samples were loaded to dryness (until all solution had passed entirely through the adsorbent). Two 10ml volumes of 0.2% aqueous TFA were passed through the cartridge, again to dryness. (This step was to wash away any weakly bound non-specific proteins.)

The sample was then eluted with 4.5ml of the 80% aqueous ACN solution containing 0.2% TFA, with collection over a ten minute period. The resin was then regenerated by a wash with one cartridge reservoir full of 100% methanol followed by a reservoir volume of the 80% aqueous ACN solution containing 0.2% TFA, and finally two reservoirs volumes of 0.2% aqueous TFA solution. After vacuum drying of the eluted sample, assay buffer was added and samples were left to stand at room temperature for 1 hour prior to the assay. Samples extracted using this method were either assayed using one of the vasopressin radioimmunoassays or counted for radioactivity.

2.3.4 Gel filtration chromatography

Gel filtration was employed to establish physical elution properties of immunoreactivity observed in some samples.

Samples were added to prepared Sephadex G-25 columns, and were eluted with a flow rate of approximately 1ml/min by gravity fed flow. All samples were eluted in 0.05M Tris-HCl (pH 7.2) buffer. Eluant was collected in 1ml fractions. Fractions were immediately assayed, either for vasopressin by radioimmunoassay, or by counting of radioactive isotope in aliquots.

Concentrated fractions were centrifuged in molecular weight cut-off filters as recommended by the manufacturers.

2.3.5 SDS-PAGE and immunoblotting experiments

Foetal muscle and liver, samples were extracted for protein using TRIzol reagent. This is a three step extraction in which DNA and RNA are isolated first. The extraction was carried out according to the supplied instructions, and the DNA and RNA fractions discarded.

Weighed portions of the freeze dried muscle samples were added to lysis buffer and left for one hour at 4°C. These samples were then homogenised by sonication for fifteen 2 second bursts at 30% of full power. MCF-7 cells were sonicated without any prior period of standing. Homogenates were then spun for ten minutes at 12,000g at 4°C, and supernatants were decanted and stored at -20°C until used.

Samples extracted using TRIzol reagent were normalised for protein using absorbance at 280nm. Samples extracted in lysis buffer were assayed for protein according to the manufacturers instructions. After protein assay, samples were mixed 1:1 with Laemmli sample buffer and heated at 70°C for ten minutes. 25µg of total protein were added to individual wells. Samples were separated by electrophoresis for 2 hours at 75V.

Separated proteins were transferred to Immobilon-P transfer membrane using Biorad electroblotting apparatus in transfer buffer. Transfers were carried out overnight at 20V at 4°C. After removal from the electroblotting apparatus,

membranes were thoroughly washed in double distilled water. Blocking was achieved by completely drying the membranes for 30 minutes at 50°C.

Primary antibodies were applied to the membranes by overnight incubation at 4°C. All antibodies were diluted in Western blotting buffer containing 5% BSA. After this, membranes were washed in Western blotting buffer for 15 minutes and three 5 minutes periods with fresh changes of buffer. Goat anti-rabbit second antibody was diluted to 1:20,000 (v/v) with Western blotting buffer containing 5% BSA, and membranes were incubated in this solution for 1 hour at room temperature. Second antibody solution was removed and the membrane was washed for 15 minutes and three periods of 5 minutes with fresh changes of buffer.

Immunoreactive proteins were visualised using chemiluminescence. The membrane was incubated in Lumi-Light Western Blotting substrate (Boehringer Mannheim) for 3 minutes. The membrane was then exposed to x-ray film.

A negative control experiment was carried out by repeating the above with the omission of primary antibody.

2.3.6 Immunohistochemistry

2.3.6.1 General procedure

After melting the paraffin wax by heating the slides for twenty minutes at 55°C, paraffin wax was removed by immersion in four changes of xylene for five minutes. Xylene was removed by washing in 100% ethanol and tissues were rehydrated by five minutes incubations in 95% ethanol (once), 90% ethanol (twice) and 70% ethanol (twice). The slides were then immersed for five minutes under running tap water.

Antigen unmasking was achieved either by microwave antigen retrieval, or trypsin digestion. Non-enzymatic methods of antigen unmasking have been shown to sometimes uncover antigens on formalin fixed paraffin embedded tissue sections not revealed by enzymatic treatment (229, 230).

Microwave antigen unmasking was performed after the tap water wash by heating the slides in a citrate solution (750µl of antigen unmasking solution in 80ml distilled water). Slides were heated for two five minute periods (1150W power - oven was set to power rating 3) or until the solution had just started to boil. After incubating the slides for twenty minutes at room temperature, slides were washed for five minutes in running tap water.

Endogenous peroxidase activity was then blocked by a twenty minute incubation in a 0.9% hydrogen peroxide solution in 100% methanol.

After being rinsed in tap water, the slides were loaded onto coverplates whilst immersed in distilled water and placed in a coverplate rack. The slides were rinsed with three fillings of the coverplate reservoirs with IHC buffer.

Where appropriate (when microwave antigen retrieval had not been carried out), 200µl of a 1mg/ml aqueous trypsin solution was added to the slides and incubated at room temperature for twenty minutes. The trypsin was removed by adding 1ml of 95% alcohol. The alcohol was then washed away by filling the reservoir three times with IHC buffer.

Non-specific binding of antibody was blocked by incubation for twenty minutes with 5% (v/v) normal goat serum in IHC buffer. After a light rinse (1ml of IHC buffer added to reservoir), the primary antibody (diluted to the correct ratio in a

10% (v/v) solution of normal goat serum) was added in a 200µl aliquot and incubated overnight at 4°C.

Slides were washed with IHC buffer (two fills of the coverplate reservoir) and the slides incubated for thirty minutes at room temperature with biotinylated second antibody. After washing (two fills of IHC buffer), the slides were incubated with an avidin-biotin enzyme complex (ABC kit) for thirty minutes at room temperature. Colour was developed after washing away excess ABC, by incubating with 200µl of the DAB kit for three to six minutes. DAB was washed away with distilled water. The slides were counter stained with haemotoxylin for 1 minute. Excess haemotoxylin was washed away with distilled water. Slides were removed from the coverplates, dehydrated through increasing concentrations of alcohol, dipped in xylene for three minutes and coverslipped.

2.3.6.2 Treatment of specific tissues

Hypothalamus and posterior pituitary tissue section antigens were retrieved by treatment with trypsin. They were incubated with negative control (normal rabbit serum) or *gonzo* antibody at 1:400 dilution of the neat serum.

Foetal human muscle tissue sections were cut in groups of five. One section was stained with hemotoxylin and eosin. Other slides were subjected to microwave antigen retrieval and stained with either normal rabbit serum at 1:100 dilution (negative control), *gonzo* antibody diluted to 1:100, *vivian* antibody at a protein concentration of 10µg/ml, or vimentin antibody, which was purchased and used in a prediluted form.

Foetal human adrenal gland sections were cut in groups of three. One section was stained with hemotoxylin and eosin. The remaining two slides were subjected to microwave antigen retrieval and tested with either normal rabbit serum at 1:100 dilution (negative control), or *gonzo* antiserum diluted to 1:100.

Adult rat adrenal gland sections were cut in groups of three. One section was stained with hemotoxylin and eosin. The remaining two slides were subjected to microwave antigen retrieval and tested with either normal rabbit serum at 1:100 dilution (negative control), or *gonzo* antiserum diluted to 1:100.

Chapter 3

Characterisation of Vasopressin Radioimmunoassay

3.1 Principles of radioimmunoassay

The detection and quantification of biologically important molecules has been a long sought after and subsequently constantly refined technique. In a clinical environment the rapid and accurate analysis of the content of a particular molecule in a biological fluid is a vital tool in the diagnosis and monitoring of many conditions, and radioimmunoassay (RIA) has proved to be an extremely versatile tool for this purpose. The first description of a radioimmunoassay was by Yalow and Berson in 1960 - a radioimmunoassay for insulin (231).

The ability to quantitatively measure a molecule in a radioimmunoassay is dependent on the creation of a standard displacement curve (or a standard curve). The "ligand", commonly the molecule of interest, is incubated with the "binder", and the "tracer" (232). The tracer is a radioactive isotope, incorporated into the molecule of interest, and the binder is a specific antibody with a high affinity for the molecule of interest. A radioimmunoassay works according to the laws of mass action - they are competition binding assays. Increased binding of unlabelled ligand leads to a proportional decrease in binding of tracer, as the two molecules bind to the same site on the antibody. By incubating known increasing concentrations of a standardised preparation of the ligand with a constant amount of tracer, separation and quantification of the bound portion of tracer from the free portion makes it possible to create a standard curve. Thus, the presence and amount of the molecule of interest

in a sample can be established by replacing standard with sample and incubating with the same amount of tracer and concentrations of binder. The distribution of the tracer between the bound and free portions will be equivalent to a concentration of standard on the standard curve. By simple extrapolation from this curve, the concentration of the molecule in the sample is found.

3.2 Characterisation of a radioimmunoassay

A radioimmunoassay can be characterised and standardised using four criteria: accuracy, precision, specificity, and sensitivity.

3.2.1 Sensitivity

Chard describes sensitivity as the “minimal detection limit of an assay”, or “the least concentration of unlabelled ligand which can be distinguished from a sample containing no unlabelled ligand” (232). This is found by comparing the standard deviation observed in samples of zero standard with the standard deviation observed in samples containing decreasing concentrations of ligand. The concentration of ligand at which the lowest possible binding of standard is higher than the highest possible binding of standard in a sample with no ligand is taken as being the sensitivity of the assay.

3.2.2 Accuracy

The accuracy of an assay is the closeness to which the estimated concentration of a sample approximates the real concentration. The most important factor in establishing an accurate assay is the existence of a standard preparation of

the ligand, the concentration and purity of which are known in themselves to a high degree of accuracy. Typically, this is found by the combination of many studies carried out in different laboratories to produce a consensus figure for a preparation or pool of material that is then be used as a standard, a benchmark by which an assay can be prepared with a high degree of confidence.

Accuracy is affected by the specificity of the binder. Any factor in the sample that alters the binding characteristics of the assay will alter the accuracy of the assay. Accuracy is also affected by the precision of an assay, but this is treated as a separate criterion and is discussed below.

Accuracy can be described in terms of the between assay variation. This is based on the measurements of aliquots from a large pool of a sample in sequential assays. The aliquot is treated as a single sample, and as such assayed alongside other unknown samples - use of a larger number of replicates within an assay would give a false impression of the variation between assays (232).

Such a measure of between assay variation is a form of quality control. Quality control allows an individual assay to be accepted or rejected on the basis of accuracy. This is done by both statistical and instinctive examination of the information provided by the quality controls to judge that the assay is working accurately and precisely inside control limits (232). Chard sets out several necessary characteristics of samples to be used for the monitoring of an assay: the samples used should contain the molecule of interest in either the buffer which the assay uses or in the biological fluid the assay is intended to evaluate; there should be enough of the pool so that it can be made into aliquots to supply controls for many months or even

years; and the pools should be chosen (or made) such that their concentrations represent high, medium, and low values in the standard curve.

3.2.3 Precision

The precision of an assay is the variation in estimated concentration with which a sample is described. It can be thought of as the reproducibility of the estimate of the molecule of interest in a sample (232). Precision can be described by the use of repeated determinations on either a quality control pool or one or more unknown samples within a single assay. Commonly, the mean and the standard deviation are used to calculate an expressed precision as a percentage of variation of the estimate from the mean.

3.2.4 Specificity

Establishing that the antibody only has affinity with the molecule of interest and not with those that have structural similarity is also important. A good example is the neurohypophyseal hormones themselves. They have very similar primary structures, differing from each other by only one or two amino acids, and so it is vital to demonstrate the specificity of an assay in order for it to be used with confidence when measuring hormone levels in samples where more than one peptide hormone may be found.

3.3 **Vasopressin radioimmunoassay**

The first RIA for vasopressin was described in 1970 (233). The problems associated with the development of a vasopressin RIA for use with blood plasma

samples can be discussed within the context of the four criteria listed to characterise assays in Section 3.2.

The generation of specific antibodies to the peptide is a first requirement and problem. Molecules of low molecular weight, like vasopressin, do not elicit a strong immune response. Although as few as six amino acids will elicit antibodies, and the peptide alone will give rise to specific antibodies (antibodies to vasopressin were first reported in 1966 (234), and early examples of are antibodies produced by animals immunised are listed in (235)), a more effective way to produce antibodies is to conjugate the peptide to a larger molecule, such as bovine serum albumin (BSA) or thyroglobulin, rendering it more antigenic (236). The antibodies used in assays described in this chapter were raised in this manner.

Another problem in vasopressin radioimmunoassay development for the assay of biological samples is the optimisation of the assay so that it is sensitive to be able to detect the peptide at the low concentrations it is normally found at. These circulating concentrations are much lower than many other larger hormones (typical resting concentrations are of the order of 2pg/ml (237)). In the event of it being impossible to produce sufficient sensitivity assay through optimisation, an effective way to get over this hurdle would be to incorporate a concentration or extraction step prior to the assay itself.

A third potential problem in assays is the interference encountered in assays of unextracted biological fluids, typically urine or blood plasma. (234, 235). Chard refers to this interference as "non-specific non-specificity" or "matrix effects" (232). This interference compromises both the sensitivity and accuracy of the assay - it commonly leads to an over estimation of the amount of the molecule being

investigated in the sample. This is ascribed to non-specific high molecular weight material in the biological fluid that does not change in response to physiological stimuli (237). Other examples of material that can interfere in the assay are heparin, salts and urea, acids and alkalis, nucleic acids and haemoglobin (232). Again, a solution to this is the extraction of the peptide prior to assay (237); an example of this is the premeasurement treatment of urine (238).

The aim of the experiments described below was to standardise and characterise another precise assay that could be carried out in parallel with, or as a substitute for, the already described assay using *tgl* antiserum. By comparing the two it was hoped that data from the two could be related in terms of accuracy. The use of more than one antibody preparation was employed to confirm the specific nature of the reported immunoreactivity in foetal muscle extracts, and so validating that the two assays were significantly comparable was important (187). The cross-reactivity of the sheep antiserum was investigated using all naturally occurring peptides that could conceivably interfere with the accuracy of a specific assay for arginine vasopressin, in order to rule out the immunoreactivity being due to the presence of a different peptide.

It should be stated that no efforts were made to redevelop the fundamental steps of the assay beyond those used as standard in the laboratory in which the experiments were carried out. The constituents of a standard assay buffer were not modified. The assay was buffered using Tris-HCl solution, but no differences were observed for the standard curves gained using this buffer from those obtained when a standard phosphate buffer was used (data not shown). Assay volume was not investigated, nor was the method for the separation of bound and free phases. Also,

the tracer was purchased from the same commercial source and was not the focus of any experimentation beyond the observation that neither the shape of the standard curves nor the sensitivity were significantly changed by adding double the amount of tracer in the same volume (data not shown).

The rationale for not optimising the sensitivity of the assay by various available methods was that it was thought that the immunoreactivity encountered during the experiments would be of a sufficiently high level to render the actual value of the lowest detectable dose irrelevant.

3.4 Results

3.4.1 Characterisation and standardisation of anti-AVP sheep antiserum s278

The titration curve for the sheep antiserum s278 is shown in Figure 3.1. Using this, the serum was diluted to 1/50000 (giving a B₀ of 35% of 5000 counts) for all future binding experiments and assays.

3.4.1.1 Accuracy

The accuracy of the assay was established using the 1st International Standard for Bioassay. Ampoules are supplied containing the freeze dried residue of 20µg of AVP. It is recommended for use for calibration of reference preparations, and as such is considered to be an accurate preparation. The accuracy of the assay was therefore confirmed by establishing that estimated concentrations of samples conformed to known concentrations of 1st International Standard in the standard. All other preparations of AVP were calibrated using this standard.

The accuracy of measurement of a sample in an $s278$ radioimmunoassay was defined by 20 different measurements of the quality control pools in separate assays. The high concentration quality control was set at 600pg/ml. The mean estimated concentration was 622 ± 75 pg/ml - a variance of 12%. The mid-point quality control was set at 150pg/ml. The mean estimated concentration was 162 ± 12 pg/ml - a variance of 7%. The low concentration quality control was set at 40pg/ml. The mean estimated concentration was 40 ± 6 pg/ml - a variance of 16%.

After these limits of accuracy were established, assays where one or more of the quality controls were outside these limits were rejected and the assays performed again.

3.4.1.2 Precision

The variation of measurement of a sample within an assay was examined by the assay of AVP in 20 replicate tubes of the same sample in one assay.

The 1st International Standard for Bioassay was used to generate three sample concentrations at different points on the standard curve. These were 800pg/ml, 400pg/ml, and 40pg/ml. The 800pg/ml sample was estimated to contain 802 ± 53 pg/ml. This is a variance of 7%. The 400pg/ml sample was estimated to contain 397 ± 23 pg/ml. This is a variance of 6%. The 40pg/ml sample was estimated to contain 43 ± 5 pg/ml. This is a variance of 13%.

3.4.1.3 Sensitivity

The sensitivity of the assay was examined by the serial dilution of AVP standard. Nine tubes were assayed for each concentration along with nine tubes containing only assay buffer. The results are shown in Figure 3.2. Figure 3.2 also shows the two lowest concentrations set against the data for the B_0 tubes. The figure demonstrates that it would not be possible to distinguish the lowest dose from the B_0 , but binding of tracer in the presence of 7.8pg/ml AVP is sufficiently different from the observed B_0 for estimates of concentration at this level to be quoted confidently. No further refinement of the sensitivity was carried out, and so it is only possible to say that the assay has a sensitivity of at least 7.8pg/ml.

3.4.1.4 Specificity

The effect of closely related peptides on the binding of AVP to an aliquot of the s278 antiserum was examined using standard preparations of peptides. The resulting displacement of binding due to antibody cross reactivity is shown in Figure 3.3.

Cross reactivity can be defined as the amount of a material required to displace 50% binding (of B_0) of the tracer to the antibody, compared to the amount of reference standard required to do the same (239). Using this definition, the cross reactivity can be expressed as a percentage of the amount of standard. Thus, the s278 antiserum has a cross reactivity of 12% with LVP, 0.1% with AVT, and less than 0.001% with oxytocin.

3.4.2 Comparison and correlation of *tg1* assay and *s278* assay

The binding curves of radioimmunoassays using the antisera *s278* and *tg1* when incubated with varying concentrations of AVP are shown in Figure 3.4.

The results of assays in which the same samples were measured are shown in Figure 3.5. Analysis of the estimated concentrations of the samples gained from the two assays by a paired Students t-test showed that there was a non-significant difference ($P>0.05$) between the two assays.

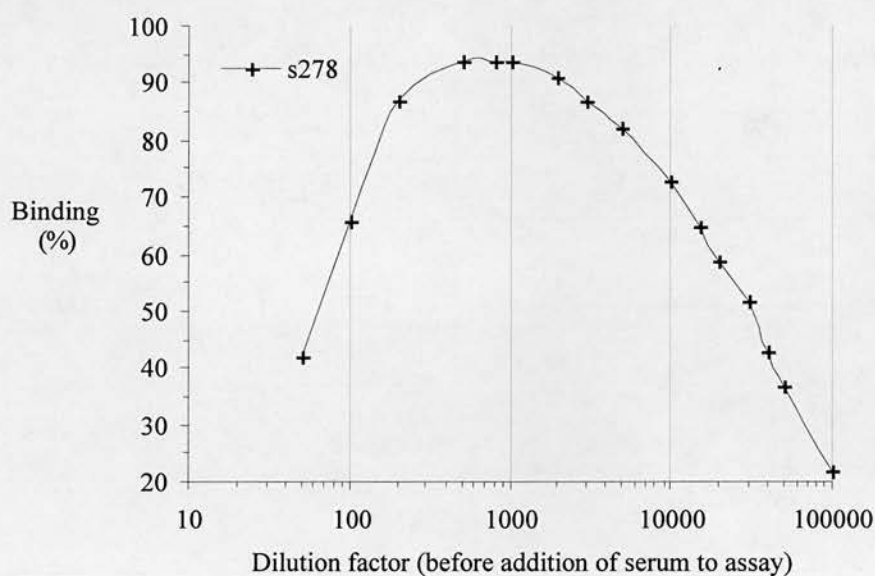
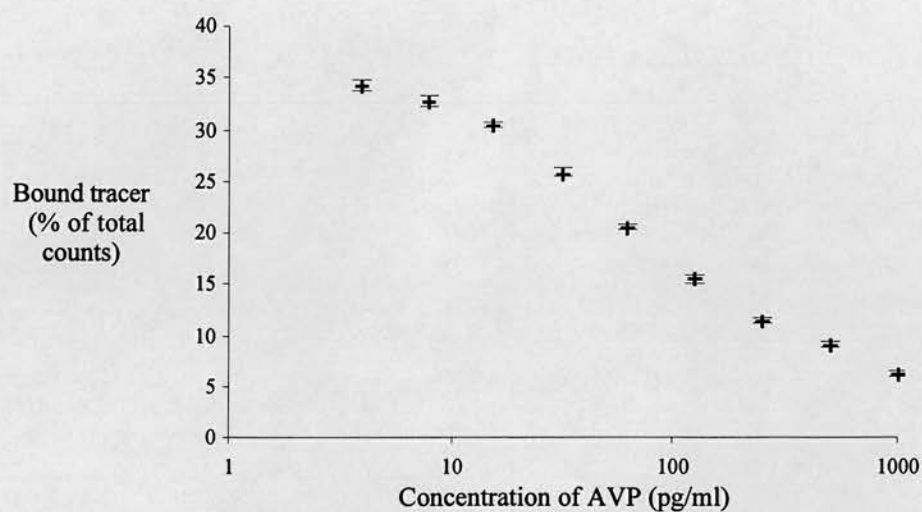


FIGURE 3.1 Titration of s278 sheep antiserum

s278 pooled sheep serum was diluted serially in assay buffer and incubated overnight at 4°C with ^{125}I -AVP (5000 counts per tube). Bound tracer was then precipitated using second antibody/PEG mixture, separated by centrifugation as for the radioimmunoassay, and counted. Binding was calculated as a percentage of the total counts. This titration was used to select the dilution at which the serum was used in the radioimmunoassay.

A



B

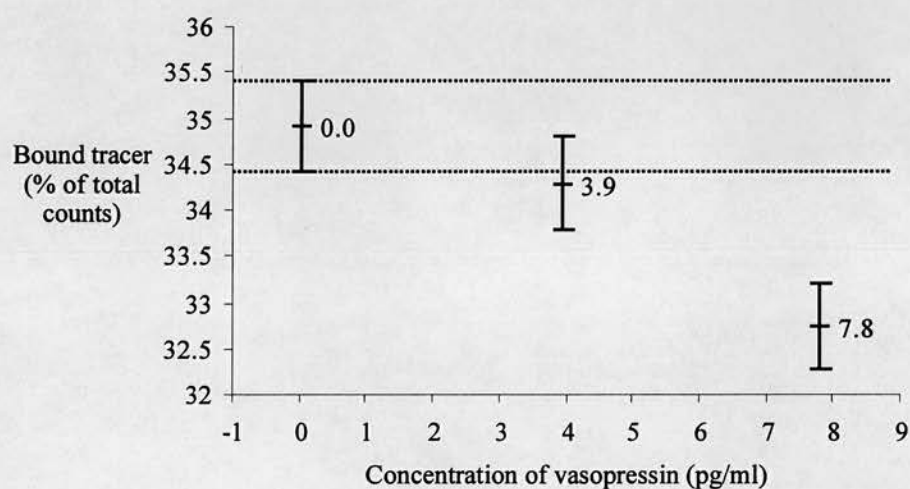


FIGURE 3.2 Sensitivity of s278 radioimmunoassay – lowest detectable dose (legend overleaf)

FIGURE 3.2 Sensitivity of *s278* radioimmunoassay – lowest detectable dose

To determine the lowest concentration of AVP that the *s278* assay could detect in the described form, a serial dilution of AVP standard was incubated with antibody and tracer as described for a radioimmunoassay ($n=9$). Bound tracer was separated as for the radioimmunoassay, and counted. The percentage of tracer bound was calculated using the total counts added.

A

Shows that the amount of tracer that was bound decreased with increased concentration of added AVP.

B

The standard deviation of percentage of bound tracer was calculated for all points and the two lowest concentrations are shown along with that of the B_0 . It can be seen that lowest concentration of AVP cannot be distinguished from the B_0 , but it is possible to do so with the next highest concentration. Sensitivity can be described, therefore, as the lowest detectable concentration of the radioimmunoassay using *s278* in the described form, and is at least 7.8pg/ml.

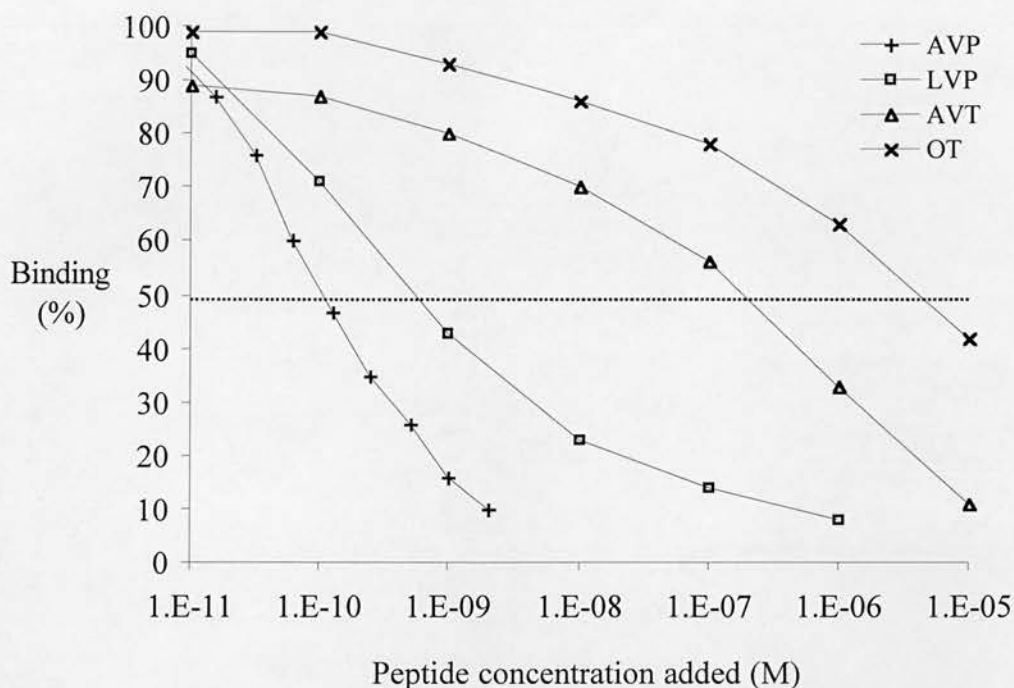


FIGURE 3.3 Cross reactions of the *s278* serum to peptides other than AVP

The ability of the *s278* antibody to detect arginine vasopressin specifically was tested using three peptides structurally very similar to AVP, and which could potentially be present in samples.

s278 antiserum diluted to 1/50000 was incubated overnight at 4°C with varying concentrations of each peptide. ^{125}I -AVP (5000 counts per tube) was then added and incubated overnight at 4°C. Bound tracer was then precipitated using second antibody/PEG mixture, separated by centrifugation, and counted. Bound tracer was compared to the amount of tracer bound in the absence of any peptide (B_0) and data are presented as percentages, signifying displacement of tracer by the non labelled peptides.

Cross reactivity is generally defined as the amount of a molecule required to displace 50% of the tracer (marked in the figure) compared to the molecule of interest, in this case AVP, and presented as a percentage. Thus, the *s278* antiserum has a cross reactivity of 12% with LVP, 0.1% with AVT, and less than 0.001% with OT.

AVP = arginine-8-vasopressin
LVP = lysine-8-vasopressin

OT = oxytocin
AVT = arginine vasotocin

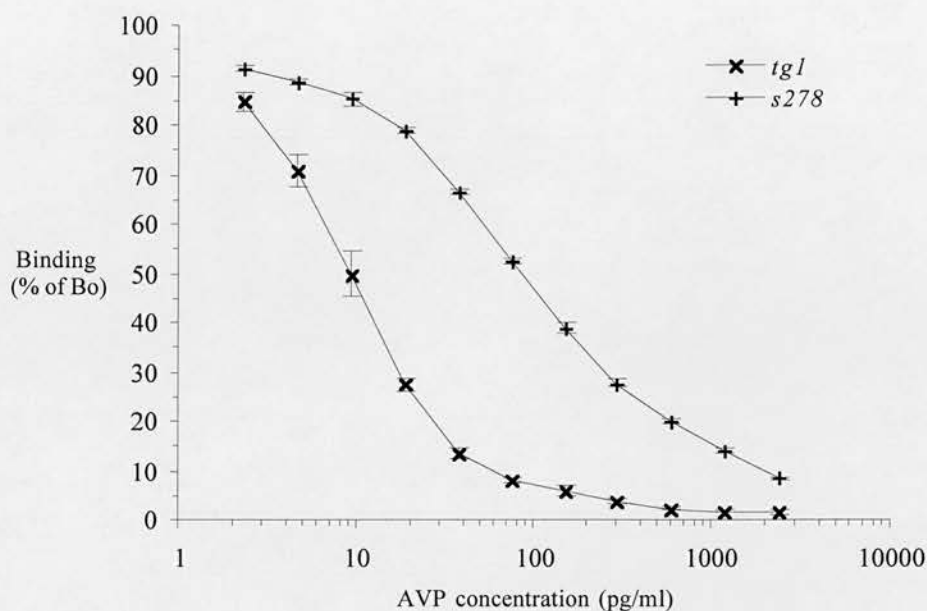
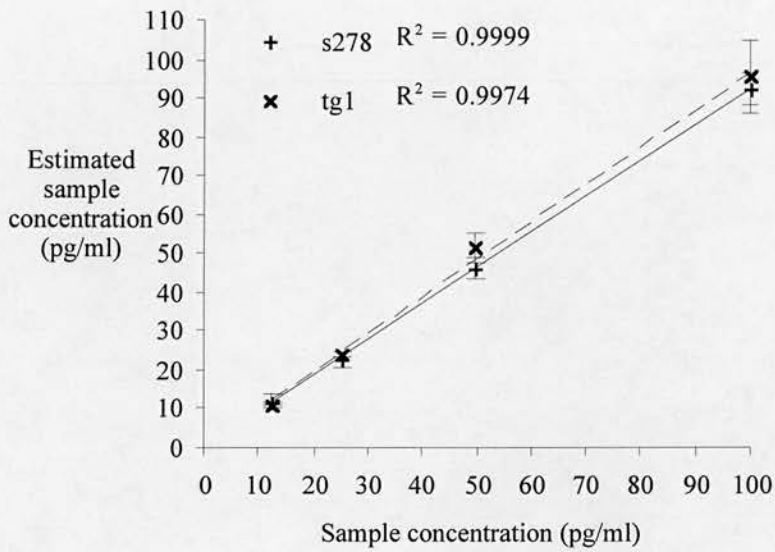


FIGURE 3.4 Sensitivity of *s278* antiserum – creation of standard curve

In order to determine the range of sensitivity of the *s278* antibody, antiserum diluted to 1/50000 with assay buffer was incubated overnight at 4°C with varying concentrations of standard AVP. ^{125}I -AVP (5000 counts per tube) was then added and incubated overnight. Bound tracer was then precipitated using second antibody/PEG mixture, separated by centrifugation, and counted. Bound tracer in the sample tubes was compared to the amount of tracer bound in the absence of AVP (B_0 , designated 100%) and data are presented as percentages, signifying displacement of tracer by the non labelled AVP.

A



B

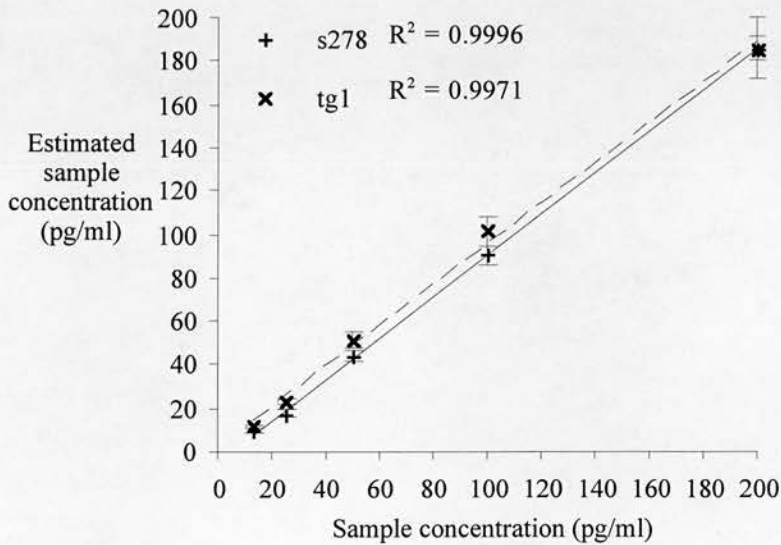


FIGURE 3.5 Comparison and correlation of *tg1* and *s278* assays

Known concentrations of standard AVP were measured as unknowns in standard radioimmunoassays using both the *tg1* and *s278* antibodies. A: 200, 100, 50, and 25pg/ml were measured. B: 100, 50, 25, and 12.5pg/ml were measured.

A paired Student t-test analysis showed a **non-significant** difference ($p > 0.05$) between the estimated doses from the two assays.

3.5 Discussion

The results in this chapter show that the sheep antiserum *s278* can be used in a radioimmunoassay for AVP. *s278* had been used to demonstrate immunoreactivity in foetal muscle previously (187), and in the interests of conformity would be used again for the same purpose. It was therefore necessary to standardise the assay.

The evaluation of the cross reactivity of the *s278* antiserum under assay conditions with peptide analogues of AVP that differ by only one or two amino acid residues, indicates that an assay involving this antiserum is suitable for assay of vasopressin under conditions where there might be other peptides in the samples, such as assays of pituitary extracts where oxytocin is present. This specificity avoids a need for further separation steps, for example the use of HPLC. Under the controlled conditions of the RIA, evaluating standard AVP diluted with assay buffer, the assay will provide the same value of estimated AVP as that supplied by the more sensitive assay using *tg1* as binder.

The within assay and between assay variations for the assay using *s278* antiserum compare favourably with the reliability of other reported radioimmunoassays for AVP (228, 238 , 240-245).

With reference to circulating physiological levels of AVP, an assay using the *s278* antiserum in the form presented cannot be used for the measurement of AVP from plasma, serum, or urine without a prior step to concentrate the peptide, because the low concentration it is found at in those fluids is lower than the sensitivity of the assay. Though it is conceivable that optimising the detection limits of the assay would permit the use of the RIA for this purpose, the *s278* assay as described should be well suited for the role of the assay of the much higher expected concentrations of

AVP from tissue extracts. In this respect, the use of *s278* antiserum assay in the estimation of high concentrations of vasopressin extracted from tissues may be preferable to using *tg1* for this purpose. Use of the *s278* assay to examine higher concentrations of AVP was considered viable and was considered a less costly alternative to the use of *tg1* antiserum to assay the anticipated high concentrations of AVP.

Chapter 4

Radioimmunoassay of Samples Containing Acetic Acid

4.1 Introduction

In Chapter 3 the characterisation of a radioimmunoassay to quantify high concentrations of AVP was described in anticipation that, according to published observations, skeletal muscle would be a source of AVP.

The starting point for the work described within this chapter is a report of high levels of specific vasopressin immunoreactivity from a novel source - foetal human skeletal muscle (187). In that report, radioimmunoassay is used in conjunction with additional techniques to demonstrate significant levels of apparently specific vasopressin immunoreactivity. Muscle tissue was homogenised in molar acetic acid; at low pH, neurohypophyseal peptides are stable and tissue enzymes are inactive, and so many of the solutions used in the homogenisation of tissues to extract peptides are deliberately acidic. The homogenate was placed in a hot water bath for at least ten minutes and centrifuged at 25,000g for an hour. The supernatant was either "assayed directly" or further purified before being assayed.

The assay of tissue extracts for AVP appears to pose slightly different problems than those encountered when attempting to estimate the concentration of AVP in biological fluids. The sensitivity of an assay should not be an issue. For example, concentrations of peptide present in posterior pituitary glands will be very high, and the ability to dilute the sample will remove the problem of detection limits as well as non-specific interference as a factor affecting the accuracy of an assay of

extract from these tissues. However, there is still a need for antibodies specifically recognising AVP. The given example of posterior pituitary glands is still relevant. Oxytocin is present in the same tissue, and so an antibody recognising this peptide as well as AVP would give an inaccurate estimate of the concentration of AVP in a sample.

A large part of the need for a radioimmunoassay in experiments establishing novel immunoreactivity is to establish parallelism in the dilution of any sample to that seen in the standard preparation. For example, when a sample is diluted by half, the assayed immunoreactivity will halve correspondingly. Non-specific interference in a high estimate of concentration does not dilute in same manner.

The aim of the experiments described in this chapter was to validate the published observations of immunoreactivity in the extracts of foetal muscle in 1M acetic acid contained in several reports (187, 217, 218).

4.2 Results

The direct assay of any sample containing 1M acetic acid resulted unfailingly in a complete lack of binding of any tracer, giving rise to a very high and imprecise estimate of AVP concentration (data not shown). Accordingly, assays were carried out on samples that had been diluted five or ten fold (yielding concentrations of 0.2M and 0.1M acetic acid in the sample). Data resulting from these assays are shown in Figure 4.1. A pattern of dilution is seen, where there is an initial high estimate for the concentration of AVP at 0.2M acetic acid, dropping to a lower estimate of peptide concentration at 0.1M and dropping to a very low level at 0.05M—commonly below the lowest detectable limits of both assays. Samples were assayed

in both the *s278* and *tg1* assay at these concentrations of acid with the aim of confirming that measurements from the two assays were comparable, but it was obvious from these assays that the measurements were markedly different, resulting in markedly different estimates of AVP content in foetal muscle samples. Potential causes of this imprecision were examined.

The assay of diluted acetic acid containing different concentrations of BSA is shown in Figure 4.2, hinting that the presence of acid in the sample is responsible for a significant portion of this apparent immunoreactivity at relatively higher concentrations of acid.

Further samples of muscle were homogenised and diluted and assayed only using the *tg1* assay. The same samples were also supplemented with standard AVP, diluted and assayed, and the resulting changes in the estimates of AVP are shown in Figures 4.3 and 4.4. The pattern of dilution was seen to change when standard AVP was introduced. At all higher concentrations of acetic acid the estimated AVP concentration never approximated those expected; only at the two lowest concentrations of acid did estimated levels of AVP conform to those that would be predicted.

Acidified extracts of foetal muscle were neutralised using 2M Tris and assayed. The resulting immunoreactivity is shown in Figure 4.5. Taking into account the blank samples, and the lack of parallelism in the dilution pattern of the neutralised samples, it was concluded that the observed immunoreactivity was due to an undefined background effect of the acetic acid.

An apparent relationship between the ratio of the weight of tissue to the volume of acid the tissue a sample is extracted in, and the observed

immunoreactivity in that sample, is shown in Figures 4.6 and 4.7. The immunoreactivity *decreased* when the ratio of the weight of tissue extracted, to volume of acid the tissue is extracted in, *increased*. This was shown to be irrespective of gestation age, as the same muscle samples were extracted with different weight/volume ratios within the same assay.

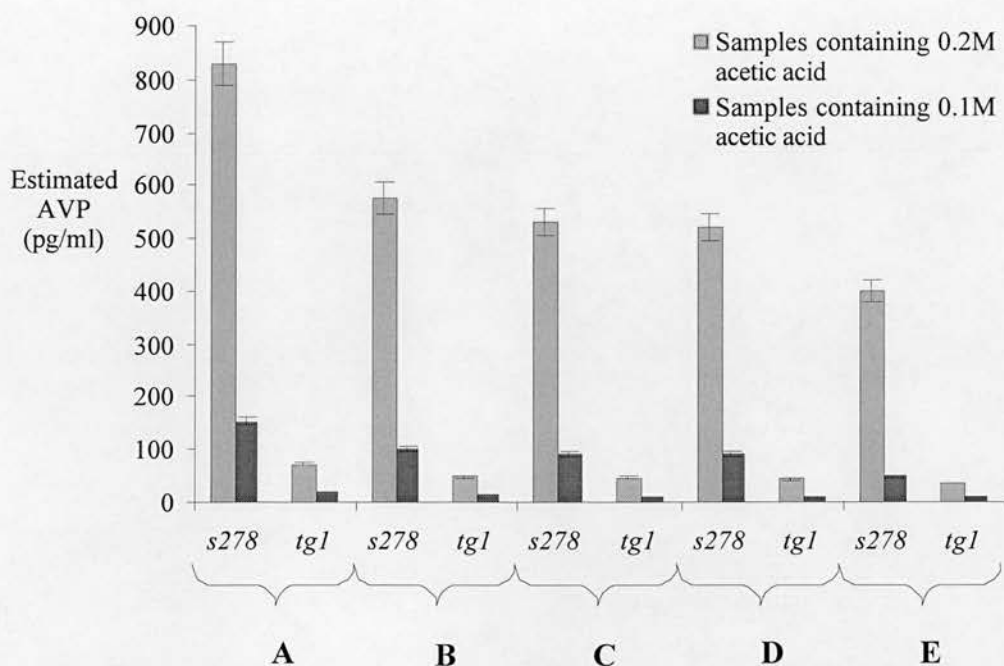


FIGURE 4.1 Effect of muscle tissue on vasopressin estimations in acetic acid extracts

Different weights of muscle tissue were each homogenised in the same volume of 1M acetic acid (8ml total volume). After centrifugation, supernatants were diluted with assay buffer so that the concentration of acetic acid was the same as that used in other experiments. The solutions were then assayed using both the *s278* and *tgl* antibodies. 0.05M acid samples were also assayed, but values were below the lowest detectable doses of both assays and so are not presented.

A – Sample was acetic acid without muscle tissue.

B – Sample was supernatant of 8ml of 1M acetic acid used to homogenise 1.2g of muscle from a 15 week old foetus

C – Sample was supernatant of 8ml of 1M acetic acid used to homogenise 2g of muscle from a 16 week old foetus

D – Sample was supernatant of 8ml of 1M acetic acid used to homogenise 2.5g of muscle from a 19 week old foetus

E – Sample was supernatant of 8ml of 1M acetic acid used to homogenise 2.5g of adult rabbit muscle

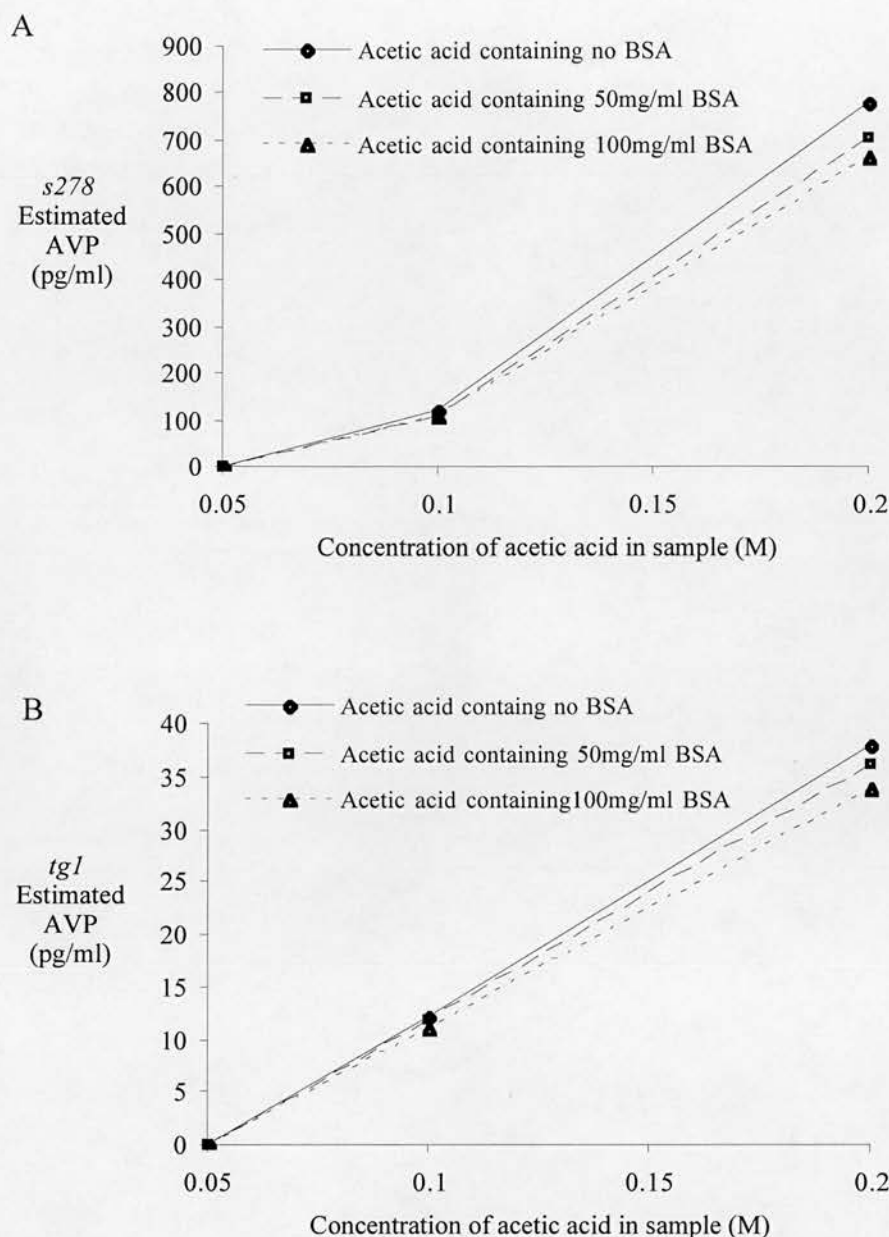
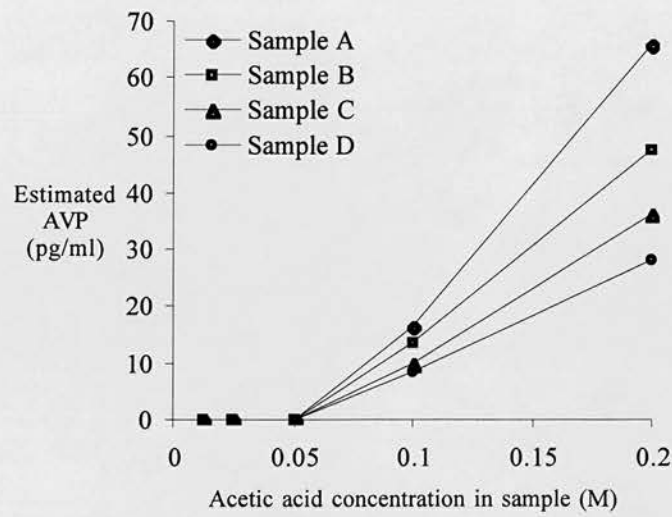


FIGURE 4.2 Demonstration of vasopressin immunoreactivity in apparent blanks
 0.2M acetic acid was prepared by dilution of 1M acetic acid using assay buffer. BSA was then dissolved in this solution to give concentrations of 100 and 50mg/ml. Further dilutions were made with assay buffer, and all the resulting solutions were assayed as samples in standard radioimmunoassays.

A: Demonstrates the immunoreactivity observed when the samples were assayed using the using the *s278* antibody.

B Demonstrates the immunoreactivity observed when the samples were assayed using the using the *tg1* antibody.

A - Adult rabbit muscle



B- Foetal human muscle

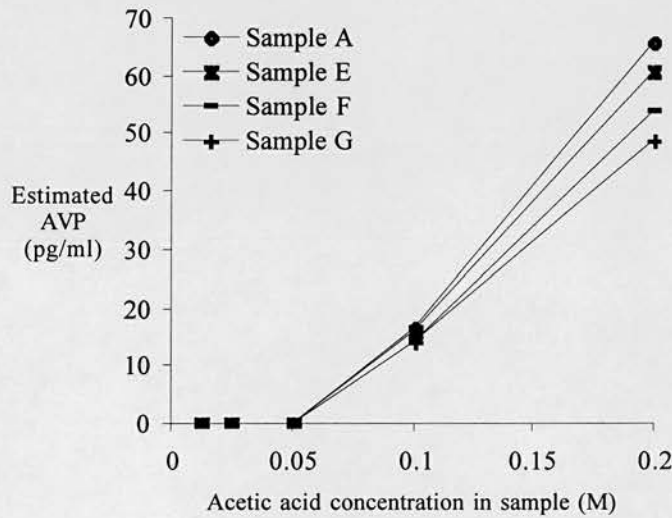


FIGURE 4.3 Effect of dilution on apparent vasopressin immunoreactivity in *tg1* radioimmunoassay
(legend overleaf)

FIGURE 4.3 Effect of dilution on apparent vasopressin immunoreactivity in *tg1* radioimmunoassay

It had been previously demonstrated that the two antisera to vasopressin had markedly different sensitivities to the presence of acetic acid in the assay. The effect of dilution with more acetic acid, and the effect of the amount of tissue in the sample was investigated. Reported values for AVP were gained using the *tg1* assay, and so no assays were done using *s278*.

Three different weights of adult rabbit muscle were homogenised in the same volume of 1M acetic acid. The same weights of muscle from a human foetus were homogenised, again in the same volume of acid. The homogenates were centrifuged, and aliquots of the supernatant were diluted with assay buffer and assayed using *tg1* antiserum.

Sample A – Acetic acid containing no tissue extract

Sample B – Aliquot of acetic acid used to extract 0.5g of adult rabbit muscle

Sample C – Aliquot of acetic acid used to extract 1g of adult rabbit muscle

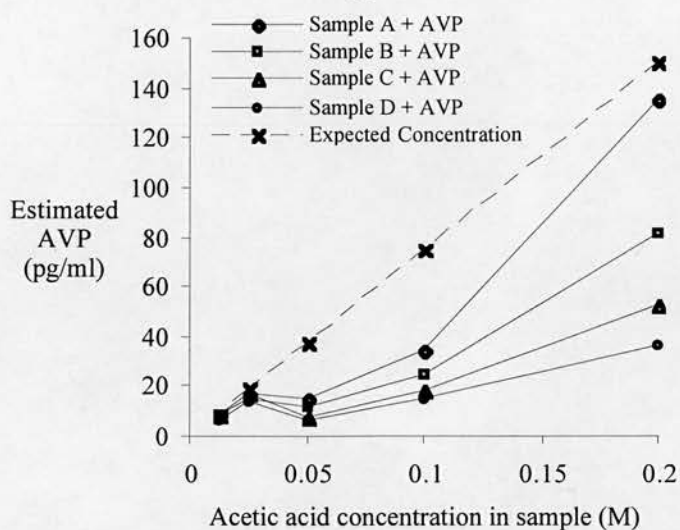
Sample D – Aliquot of acetic acid used to extract 1.5g of adult rabbit muscle

Sample E – Aliquot of acetic acid used to extract 0.5g of foetal human muscle

Sample F – Aliquot of acetic acid used to extract 1g of foetal human muscle

Sample G – Aliquot of acetic acid used to extract 1.5g foetal human muscle

A - Adult rabbit muscle supplemented with AVP



B - Foetal human muscle supplemented with AVP

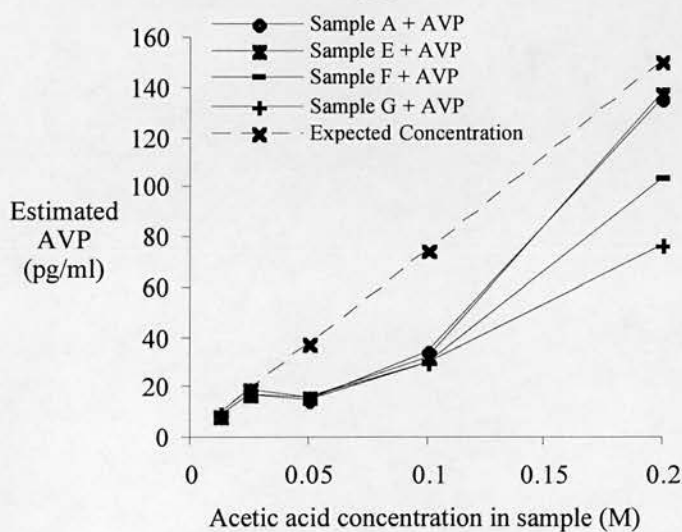


FIGURE 4.4 Effect of acetic acid on detected vasopressin immunoreactivity in samples supplemented with AVP using the *tg1* radioimmunoassay (legend overleaf)

FIGURE 4.4 Effect of acetic acid on detected vasopressin immunoreactivity in samples supplemented with AVP using the *tg1* radioimmunoassay

It had been previously demonstrated that the two antisera to vasopressin had markedly different sensitivities to the presence of acetic acid in the assay. The effect of dilution with more acetic acid, and the effect of the amount of tissue in the sample was investigated. Published values for AVP in foetal muscle were gained using the *tg1* assay of acetic acid, and so no assays were done using *s278* to investigate this effect.

Three different weights of adult rabbit muscle were homogenised in the same volume of 1M acetic acid. The same weights of muscle from a human foetus were homogenised, again in the same volume of acid. The homogenates were centrifuged, and 1ml aliquots of the supernatant were supplemented with 750pg of standard AVP. The samples were diluted with assay buffer and assayed using *tg1* antiserum.

Sample A – Acetic acid with no tissue extraction

Sample B – Aliquot of acetic acid used to extract 0.5g of adult rabbit muscle

Sample C – Aliquot of acetic acid used to extract 1g of adult rabbit muscle

Sample D – Aliquot of acetic acid used to extract 1.5g of adult rabbit muscle

Sample E – Aliquot of acetic acid used to extract 0.5g of foetal human muscle

Sample F – Aliquot of acetic acid used to extract 1g of foetal human muscle

Sample G – Aliquot of acetic acid used to extract 1.5g foetal human muscle

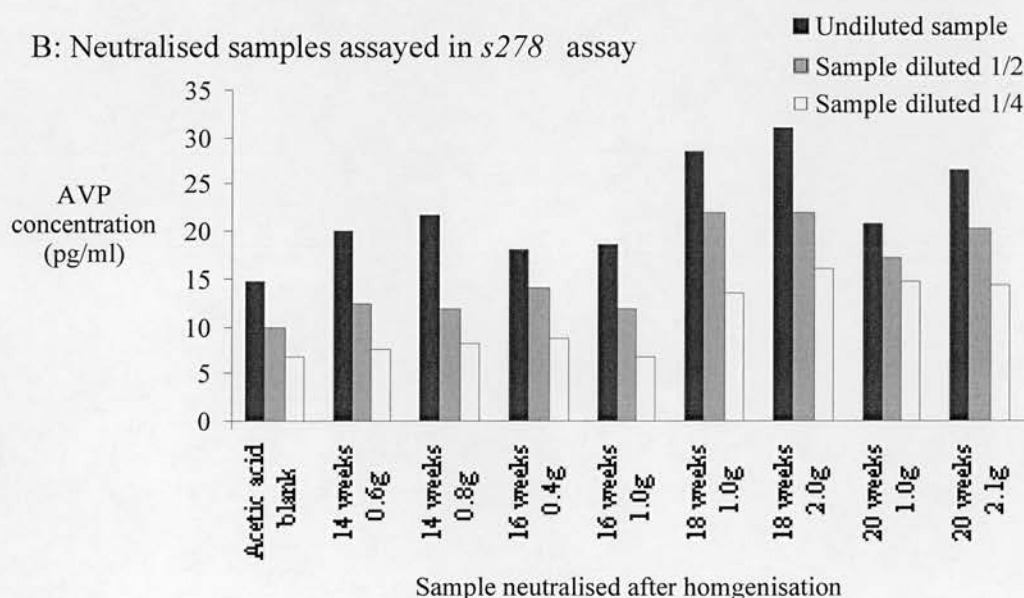
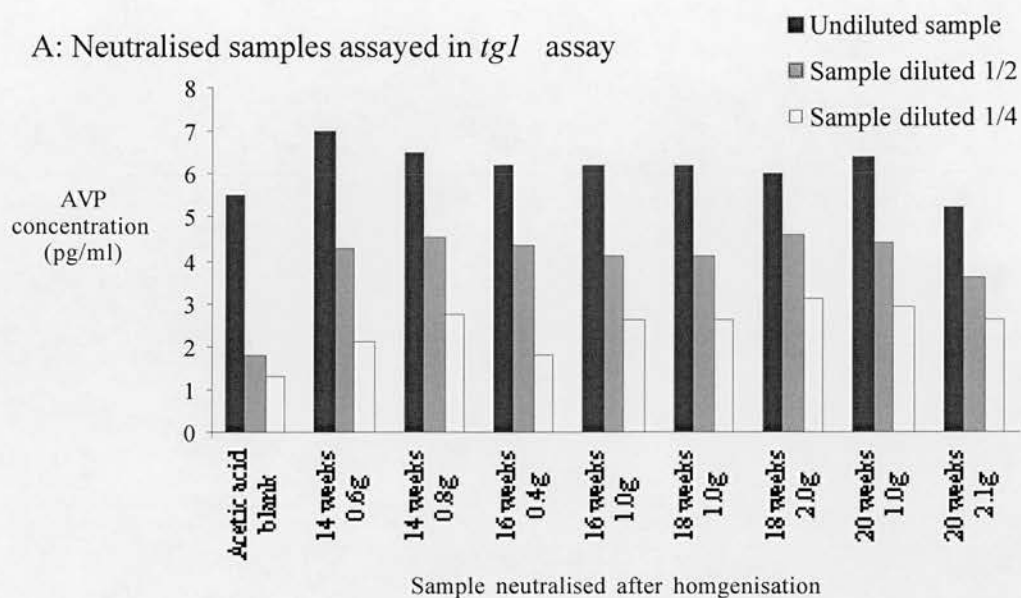
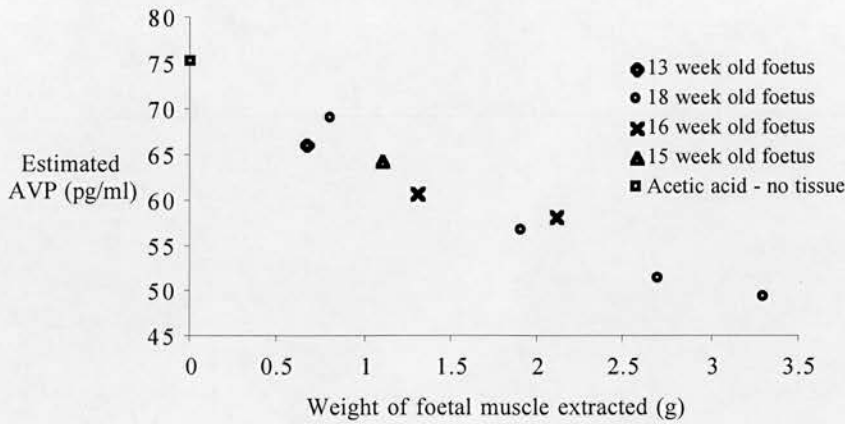


FIGURE 4.5 Effect of neutralisation of acetic acid on immunoreactivity of foetal skeletal muscle extracts

Samples of foetal muscle representative of different ages of midtrimester gestation were weighed and all homogenised in the same volume of 1M acetic acid (10 ml). After centrifugation, supernatants were neutralised (to pH 7.2) with 2M Tris. Undiluted and diluted samples (diluted with assay buffer) were assayed using both *tg1* and *s278* antisera.

A- Assay of 0.2M acetic acid foetal muscle extracts



B - Assay of 0.1M acetic acid foetal muscle extracts

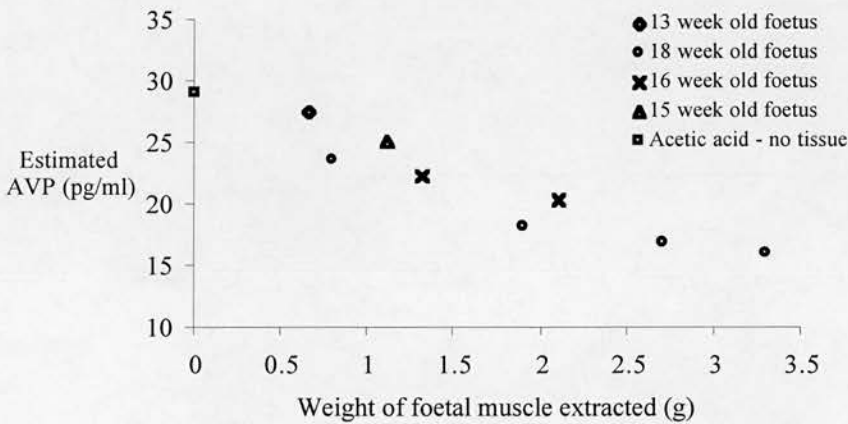


FIGURE 4.6 Effect of increasing the amount of tissue extracted in the same volume of acetic acid

Different weights of skeletal muscle from four differently aged fetuses were homogenised in the same volume of 1M acetic acid. After centrifugation, aliquots of the supernatant were diluted with assay buffer to give concentrations of the acid of 0.1M and 0.05M. These samples were assayed using the *tg1* antibody.

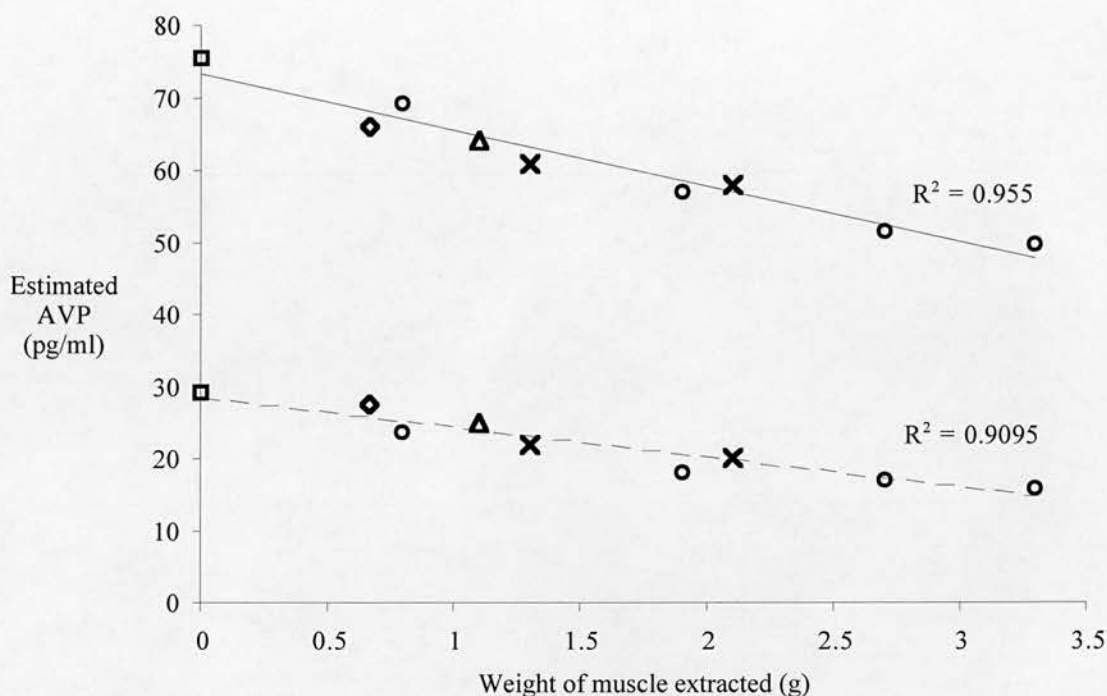


FIGURE 4.7 Effect on immunoreactivity in a sample of varying the amount of the same tissue homogenised in the same volume of acetic acid

Different weights of skeletal muscle from four differently aged foetuses were homogenised in the same volume of 1M acetic acid. After centrifugation, aliquots of the supernatant were diluted with assay buffer to give concentrations of the acid of 0.1M and 0.05M. These samples were assayed using the *tg1* antibody.

For the purposes of demonstrating a correlation, the two sets of data shown separately in Figure 4.6 were combined on a single graph. All points were treated as a single group. Linear regression analysis imposed the best fit lines and correlation coefficients as shown.

4.3 Discussion

The data from assays of AVP in tissue extracts containing 1M acetic acid demonstrate that justifying the quantification of AVP using this method is problematic. The data presented contradict the theory that it is possible to accurately measure AVP in samples containing 0.2M or 0.1M acetic acid.

Interpreted literally, Figures 4.1, 4.2, and 4.3 show that not only foetal muscle but adult rabbit muscle and even acetic acid (apparently containing nothing more than double distilled water and a small volume of glacial acetic acid, with an assayed purity of 99.7%) contain high amounts of AVP. Further, the more muscle homogenised per unit volume of acid, the less AVP the resulting homogenate contains.

These conclusions are clearly illogical. The traditional method for detecting non-specific interference of any type is the lack of parallel dilution when comparing standard and the sample (232). Data gained from the assays of acetic acid containing only BSA display just that. The obvious conclusion to be drawn is that the immunoreactivity is nothing more than acid or acetate interference, which decreases with the dilution of acid in the extraction medium. The effect of the acid is ameliorated by an increase in concentration of a factor provided not only by foetal muscle but adult rabbit muscle, suggesting that this factor is not species or developmentally specific and may simply be something as mundane as a buffering effect of soluble protein from the tissue in the acetic acid extract, explaining why increasing the amount of any tissue extracted in the same volume of molar acetic acid decreases the apparent AVP concentration.

Further, the selection of the concentration of AVP used in experiments described in Figure 4.4 was made with reference to the data of Smith and co-workers, so that it would be of a comparable scale to that apparently seen in their samples (187). All the data from assay of muscle extracts where no exogenous AVP has been added suggest that supplementation of samples with standard AVP does not significantly increase AVP levels already present in any extract.

The acid interference in a vasopressin radioimmunoassay has been noted by Chard, where an inappropriately high concentration of vasopressin was observed in an assay of plasma extracted using glass beads. The acid used in the extraction had not been adequately removed prior to addition of the samples to the assay (235). Neutralisation of acetic acid completely removed the immunoreactivity observed in acidic samples (Figure 4.5). The low concentrations in muscle samples were no higher than that seen in blank samples. Moreover, the estimations provided by *tg1* and *s278* assays were significantly different – a paired Students t-test generated a p value of less than 0.001. In assays of standard AVP described in Chapter 2, this was not observed. Given this, it is concluded that any concentration of AVP yielded from the homogenisation of foetal human muscle in those experiments was too low to be detectable using either assay. Certainly, extraction of twice the weight of muscle in the same volume did not give rise to twice the amount of AVP in the sample observed.

The relationship between gestation age and immunoreactive AVP described by Smith and colleagues was constructed from data provided by the "direct assay" of acetic acid extracts of foetal muscle (187). Most of the data from which the graph which displays this relationship was constructed are not available, but those that are

were examined, and are reproduced in Appendix A (pages 1-3 of Appendix A are reproduced, with permission, from the notebook of R. Stephen, Chief Technician, Department of Child Life and Health, University of Edinburgh Medical School, Edinburgh, UK). Also reproduced is a summary page where some of the results are collated (Appendix A, page 4, reproduced, with permission from the notes of R. Stephen).

The data shown are figures for immunoreactivity apparently based on the assay of samples that have been diluted 1/5, 1/10, and 1/20 dilutions of acid i.e. 0.2M, 0.1M and 0.05M (the dilutions are marked at the tops of the columns on the left of each page, together with the note that the assay uses *tgI*). It is apparent that all 75 samples contain significant amounts of AVP immunoreactivity, and the pattern of dilution is similar to data obtained from *tgI* assays performed for this thesis. No data is shown for the assay of samples diluted to concentrations of acetic acid extract lower than 0.05M.

The summary page lists some of the samples that apparently are foetal in origin. They are identifiable as being samples from assays in the notebook by their autopsy number and the tube number, and are indicated. These samples all have three lower numbers underneath each of the dilutions, and three large numbers to the right hand side, which are corrected values for the concentration in the sample; the lower numbers are multiplied by 5, 10 or 20 where appropriate. In some cases, these corrected values seem to demonstrate a halving dilution relationship between the samples. However, the values have *already* been corrected - they should be approximately the same after the *first* correction. It is not known how the figures in the third column of the summary page (titled pg/ml) were arrived at. The value for

this immunoreactivity is used to calculate a figure for the AVP content per gram of muscle by taking in to account the volume the muscle was extracted in, and the weight of muscle extracted. These figures, unless corrected in some way that is not noted in the sheets, originate from data that has been demonstrated by experiments described in this chapter to be, at best, inaccurate, and are used as published evidence of AVP-ir in foetal skeletal muscle.

Without knowledge of the interference effect of the acetic acid in the *tgl* assay, a series of extractions where an increase of gestation age is mirrored by an increase in the weight of muscle sample used (perhaps caused by the availability of a larger source of material given the growth of a foetus with time), but not by an increase in the volume of acid in which the muscle is extracted, would give rise to data suggestive of a relationship between gestation age and immunoreactivity specific to AVP when the extracts are assayed and corrected for these factors. Figure 4.6 shows that less tissue extracted per unit volume causes higher apparent immunoreactivity. Correction for the amount of tissue used in each extraction would cause a further widening of the difference in content per unit weight between different weights of extracted sample.

Figure 4.4 shows that direct information about the concentration of vasopressin in a sample can only be obtained by assaying for AVP in highly diluted molar acetic acid extracts. A titration of the effect of acid on the assay was not carried out, but at the very least it can be said that any attempt to use the data from RIA of acetic acid extracted tissues to provide a single figure of vasopressin content is complicated by the use of estimations provided by any assay of a sample containing acetic acid concentrations of 0.05M or above.

An obvious explanation for the failure to measure AVP in extracts from foetal muscle is that homogenisation did not liberate vasopressin present in the tissue due to retention of AVP by a specific or non-specific binding factor. A way of demonstrating that the homogenisation was effective would be to homogenise muscle with exogenous AVP included before homogenisation and demonstrate retention of AVP, and experiments where this was done are presented in this thesis.

However, boiling and homogenisation were carried out with the aim of completely dissolving the tissue and removing the activity of any factor acting to reduce AVP levels in the muscle tissue. The method of extraction used for these experiments is essentially the same as that described by Smith and co-workers. One difference in the present study was the use of a lower centrifugation force in the centrifugation of tissue homogenates (12,000g compared to the 20,000g used by Smith *et al*). But this difference strengthens the conclusion that a significant proportion of the immunoreactivity seen in the RIA is non-specific. Higher g-forces would reduce the general protein concentration in the extract supernatant, resulting in higher levels of apparent immunoreactivity. The pattern that is observed in homogenisations of different weights of the same muscle sample (Figures 4.6 and 4.7) would be unchanged.

In conclusion, it was not possible to demonstrate genuine AVP immunoreactivity in the assay of tissue extracts in which concentrations of acetic acid were present that interfered with the accuracy of the assay. Extracts containing 1M acetic acid had to be diluted more than forty times to demonstrate supplemented immunoreactivity that diluted parallel with a standard curve from an AVP radioimmunoassay.

However, the *tg1* assay was set up according to instructions so that it would have a lowest detectable limit of 1.56pg/ml of AVP. Concentrations of less than 60pg/ml in the original extract could not then be reliably demonstrated using the *tg1* assay. In this respect, the use of an extraction step was indicated to determine whether there were concentrations of vasopressin in foetal muscle that were much lower than those reported by Smith and co-workers, but which may nonetheless be of physiological significance in the maturation of foetal skeletal muscle.

Chapter 5

Solid Phase Extraction of Vasopressin From Homogenised Tissues

5.1 Introduction

The extraction of vasopressin from tissue uses the same principles and techniques as the extraction of vasopressin from biological fluids. Direct, accurate measurement of peptides in blood plasma and serum is compromised by the presence of non-specific interference altering the estimated concentration, as well as the enzymes present in many fluids and tissues from normal and pregnant individuals that have shown to be capable of degrading peptides (246). Thus, an extraction step prior to an assay is advocated by many investigators in many cases (241, 247-249).

The advantages of extraction of AVP for assay from biological fluid samples apply equally to tissues. Extraction from a tissue is also obviously vital in order to liberate the peptide from the tissues, preferably into a stable environment. The removal of accompanying interfering matter or degrading enzymes allows more accurate estimations. Concentration of the sample provides an increased sensitivity and will allow dilution of the sample to establish parallelism, thus conveying a quality of specificity to the immunoreactivity.

The practical criteria for an extraction procedure in a clinical situation are set out by Chard (232). Although the need for rapid throughput of samples is less during a laboratory investigation, these criteria can be applied to all extraction procedures. The extraction must be quick and simple, to allow a large number of samples to be

processed in the time available. The extraction should be specific, that is the sample should be concentrated into a volume considerably smaller than that of the original, but at the same time it should not concentrate the interfering factors it is sought to remove. The recovery of the extraction should be better than 50%, with good reproducibility. Chard is quite adamant on this, and he reiterates that it is much more desirable to have an extraction that reliably and repeatedly extracts with an efficiency of 60% than one that only variably extracts with an efficiency of 90%. The extraction should not affect the binding properties of the target of the extraction, and the reagents and materials used should be readily available and have little variety between batches.

Various methods have been used for the extraction of peptides. Typically, either acetic acid or hydrochloric acid is used as an extraction solvent, as neurohypophyseal peptides are stable at lower pHs (3). Tables 5.1 and 5.2 show examples of different methods of extraction. Recently, the methods of choice for the extraction of peptides have been limited to either Sephadex G-25 or G-50 gel filtration or solid phase extraction. Gel filtration elutes molecules of higher molecular weight before the lower weight peptides of interest are eluted. Also, because there is no loss of sample between homogenisation and assay, it can be assumed that the profile obtained is a good reflection of the constituents of the tissue extract.

Solid phase extraction uses the specific physical (but not chemical) binding of molecules to an adsorbent, allowing other non-specific substances to be washed away. The bound peptide is then eluted, thereby making it available for assay. As can be seen from Table 5.2, octadecasilyl silica (C18-silica) is the most commonly used

adsorbent for the extraction of peptides. C18-silica has been used in HPLC separations of peptides (250, 251), and in the solid phase extraction of peptides from biological fluids (252, 253). Based on the work of Bennett, the same procedure was used to extract pg amounts of vasopressin from biological fluids (LaRoche and North were the first to describe use of this method with neurohypophysial peptides (249), also (241, 254, 255)). Recoveries obtained using this method are typically quoted as being 70-85%.

The use of C8-silica in the extraction of vasopressin from plasma has also been described (228). Van de Heijning and co-workers found that C8-silica extraction was a more convenient and consistent method of extraction than other solid phase extraction media. Recoveries of 85-96% were achieved, with an apparent lower retention of interfering factors on the columns. Based on the data of Van de Heijning and co-workers, it was decided to use 500mg C8-silica cartridges for the solid phase extraction of vasopressin from tissues.

Experiments described in the first part of this chapter were carried out in order to evaluate and validate the use of C8-silica cartridges as part of a procedure to extract vasopressin at high or low doses, and as a method of removing the acetic acid interference encountered in the direct assay of tissues homogenised in 1M acetic acid, described in Chapter 4.

Foetal human skeletal muscle was extracted using the established method to attempt to demonstrate the presence of vasopressin as reported by Smith and co-workers (187). Foetal human pituitary and adrenal glands and rat adult adrenal glands were extracted in the same manner as positive controls. Adult rabbit skeletal muscle was used as negative control tissue.

Fluid	Extraction medium	Extraction efficiency	Reference
Urine	Vycor glass	75%	(269)
Blood plasma	Bentonite	52-93%	(270)
	Acetone/ether	67-70%	(271)
	Florisil	93%	(272)
	C18 SPE	85%	(249)

TABLE 5.1 Methods of extraction of vasopressin from biological fluids

When urine or plasma are assayed directly, a high background is frequently observed and so various media have been used to extract vasopressin from fluids to facilitate accurate assay.

Tissue	Extraction medium	Recovery efficiency	Homogenisation solution	Post extraction treatment	Reference
Pineal gland	Acetone/ether	67-70%	Acetic acid	Chromatography, gel filtration, RIA	(109)
Sympathetic nervous tissue	C18 SPE	85%	50mM HCl	Gel filtration and RIA	(120)
Blood vessels	C18 SPE	85%	1M HCl	RIA	(114)
Testis	C18 SPE	85%	0.1M Acetic acid	Gel filtration and RIA	(139)
Pancreas	C18 SPE	85%	1.0M Acetic acid	RIA	(153)
Adrenal gland	C18 SPE	85%	1.0M Acetic acid	Gel filtration and RIA	(138)
	G-50 chromatography	n/a	1.0M Acetic acid	Gel filtration and RIA	(159)
Foetal skeletal muscle	None	n/a	1.0M Acetic acid	Centrifugation and direct RIA, HPLC	(187)

TABLE 5.2 Methods of vasopressin extraction from tissues

Different tissues have been reported to contain high levels of vasopressin immunoreactivity after homogenisation and extraction. Further work to establish physical characteristics of the immunoreactivity was done in some cases.

n/a = not applicable to this method

5.2 Results

The data for the standardisation of the C8-silica cartridge extraction step are summarised in Figure 5.1. When vasopressin was extracted from acetic acid, a consistent recovery over 80% of amounts of vasopressin ranging from 10pg to 10ng was achieved.

When a concentration of vasopressin selected to be equivalent to reported doses in foetal muscle was passed through a C8-silica cartridge after addition to the supernatant of homogenised muscle (both foetal human muscle and adult rabbit muscle), this high recovery dropped, and such a drop in recovery could be related to the ratio of the amount of muscle extracted to the volume of acid used in the homogenisation (Figure 5.2). A reduced recovery was also seen when a similar amount of standard vasopressin was added to the acetic acid prior to its use in the homogenisation of a muscle sample, and so all muscle samples were extracted using 1g of tissue and 10g of acetic acid to prevent variation in the extraction efficiency of different samples.

When lower amounts of vasopressin, 100pg and 50pg, were added, estimates of the immunoreactivity were actually higher than those obtained from the C8-silica cartridge extraction of equivalent amount of vasopressin in 1M acetic acid. Similarly, low levels of vasopressin immunoreactivity were detected in the radioimmunoassay of C8-silica cartridge extracts of foetal muscle, and the data from these assays are summarised in Figure 5.3. The concentrations of the immunoreactivity was rarely ever high enough to allow parallel serial dilution of the sample, and the concentrations shown are estimations of the undiluted sample in the assay buffer used to dissolve the dried pellet of the C8-silica extraction.

Parallelism *was* established in the assay of C8-silica cartridge extracted foetal human adrenal and posterior pituitary glands. The content of vasopressin in foetal human adrenal and posterior pituitary glands are summarised in Figures 5.4 and 5.5.

Sephadex G-25 chromatography was used to characterise the mobility of immunoreactivity found in extracts of tissue. Using synthetic AVP, tritiated synthetic AVP, and AVP from a foetal posterior pituitary gland extract, it was possible to obtain a consistent peak of vasopressin immunoreactivity at one volume elution past the void volume (Figures 5.6).

No immunoreactivity or radioactivity were observed in the elution of synthetic vasopressin and ^3H -AVP just after the elution of the void volume, but displacement of tracer binding was apparent just after the void volume in the elution of pituitary gland extract, and when tritiated vasopressin was added to foetal human muscle extract, a small peak of activity was seen at the same volume of elution (Figure 5.6B), showing that it is possible for AVP to interact with higher weight molecules that cause it to be eluted much earlier than 'free' vasopressin.

Peaks of immunoreactivity were found at the void volume when extracts from foetal human adrenal glands were loaded onto the Sephadex G-25 column. The subsequent analysis of a pooled sample of foetal adrenal glands is shown in Figure 5.7.

This early peak of immunoreactivity was also observed in the elution of acid extracts from individual muscle samples. Of six individual muscle samples examined, two did not show any immunoreactivity. Four others displayed immunoreactivity after the elution of one void volume. However, no later peak of immunoreactivity could be detected (data not shown). It was attempted to generate a

peak of immunoreactivity at the same volume as that found with synthetic, tritiated or foetal pituitary AVP using larger amounts of pooled extracts of muscle and the results of this are shown in Figure 5.8. A very small level of vasopressin is demonstrated at both the void volume and the standard elution volume of vasopressin.

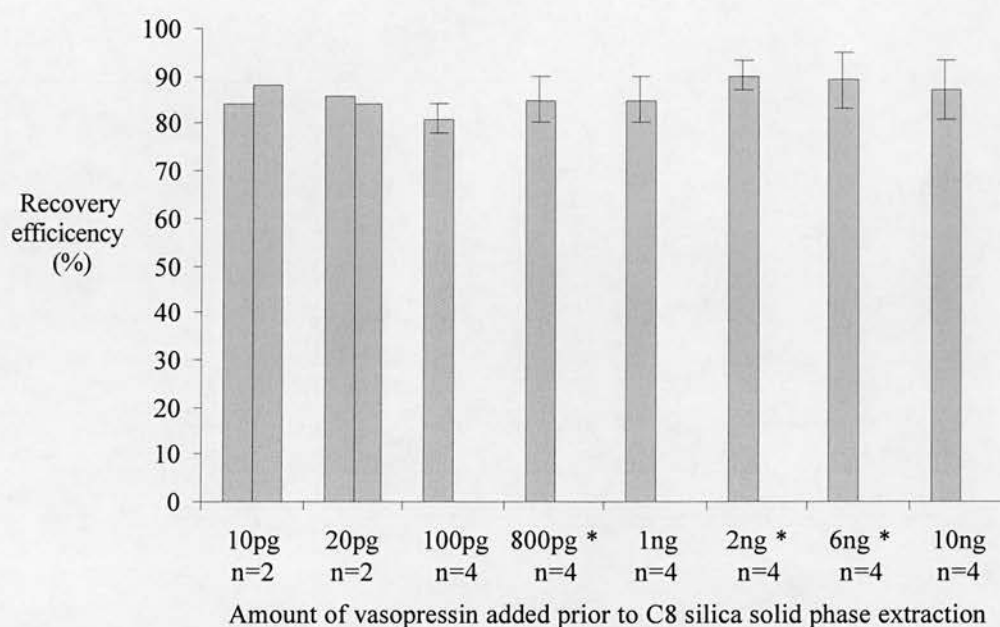
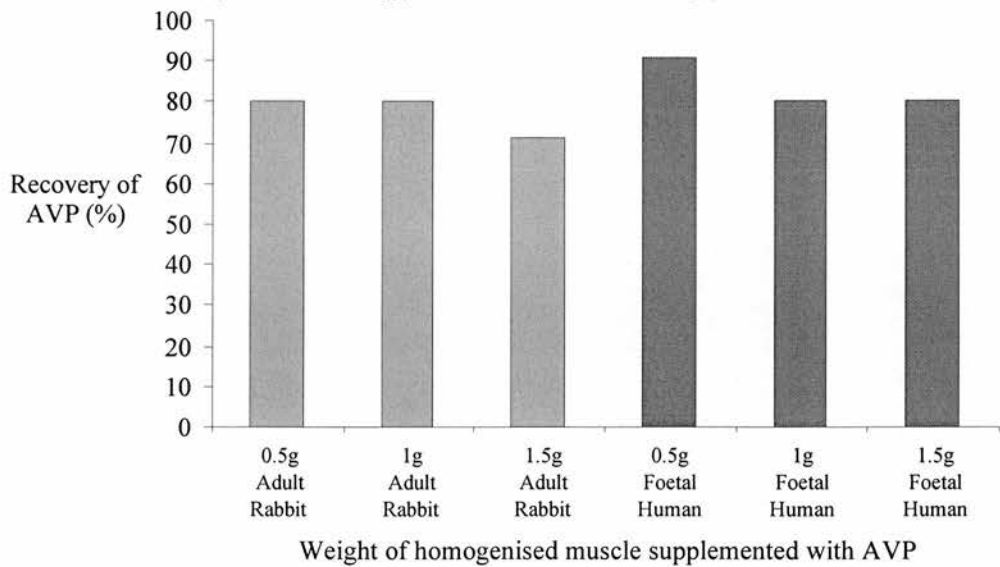


FIGURE 5.1 Physical recovery of AVP after solid phase extraction using C8-silica cartridges

Known amounts of AVP were added to 9ml aliquots of molar acetic acid and extracted using C8 silica cartridges. Eluates were either assayed using the *s278* or *tg1* antibody, or counted when ^3H -AVP was used. Recovery efficiencies are presented as percentages of total amount of AVP added as determined by RIA or data from counted beta radiation.

* indicates use of ^3H -AVP in these experiments

A - Post-homogenisation supplementation with 750pg AVP



B - Pre-homogenisation supplementation with varying amounts of AVP

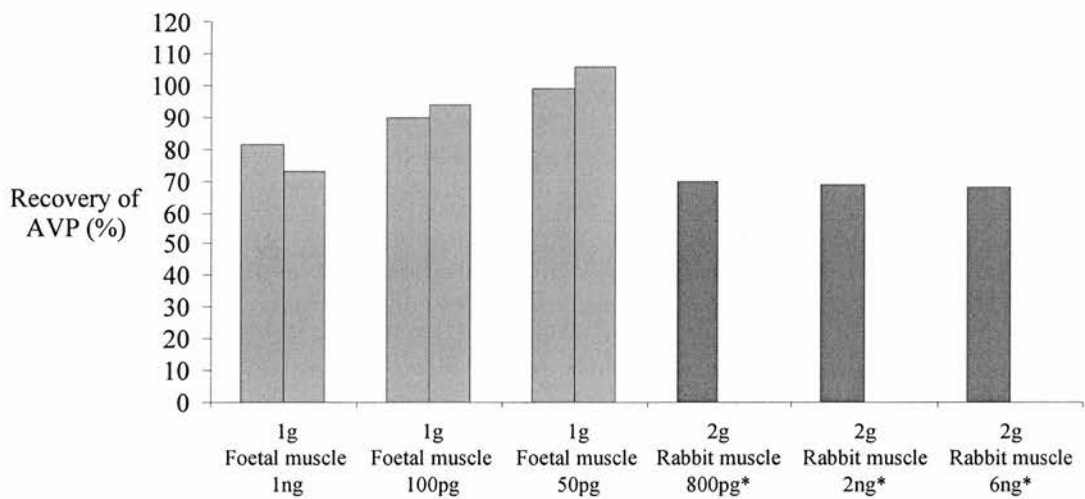


FIGURE 5.2 Effect of weight of tissue in extract on efficiency of recovery of AVP by C8-silica cartridges
(legend overleaf)

FIGURE 5.2 Effect of weight of tissue in extract on efficiency of recovery of AVP by C8-silica cartridges

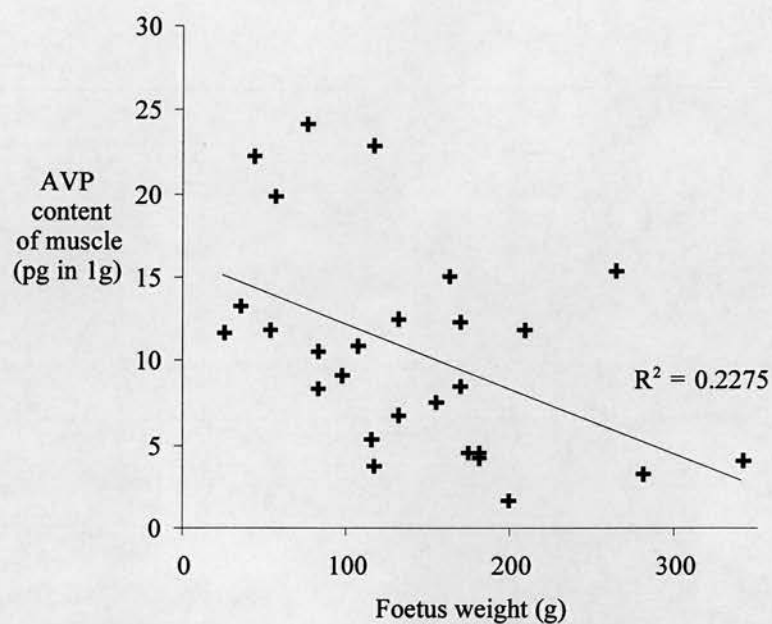
The effect of the presence of muscle tissue on recovery of AVP from acetic acid was investigated.

A Different weights of muscle were homogenised in the same volume of 1M acetic acid (9ml). After centrifugation, the supernatant was supplemented with 750pg of standard AVP (as determined by the direct radioimmunoassay of the AVP solution), thoroughly mixed, and the AVP extracted using C8-silica cartridges.

B Aliquots of known concentrations of AVP (determined by direct radioimmunoassay of the standard solution) were added to a set volume of acetic acid (10ml). This was then used to homogenise samples of foetal muscle and rabbit muscle that had been divided into equal weighed portions.

* indicates use of ^3H -AVP in these experiments

A



B

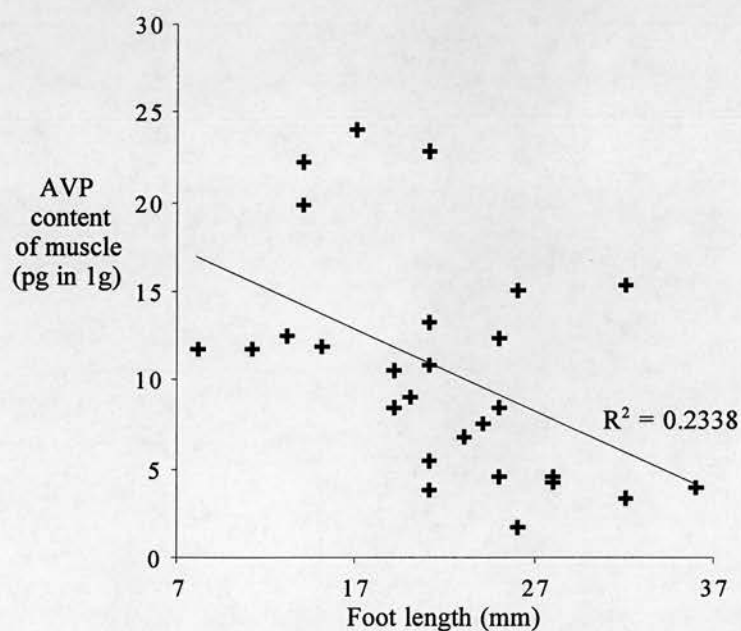


FIGURE 5.3 Relationship between gestation age and vasopressin level in foetal muscle samples
(legend overleaf)

FIGURE 5.3 Relationship between gestation age and vasopressin level in foetal muscle samples

To determine the levels of vasopressin in foetal muscle, 1g portions of muscle from foetuses of varying gestation ages were homogenised in the same volume of 1M acetic acid (10ml). After centrifugation, supernatants were extracted using C8-silica cartridges. The resulting samples were raised up in assay buffer and assayed using s278 antiserum. Measurements of AVP in the samples are shown plotted against weight (A) or foot length (B) of the foetus to assess any relationship between development and measured immunoreactivity.

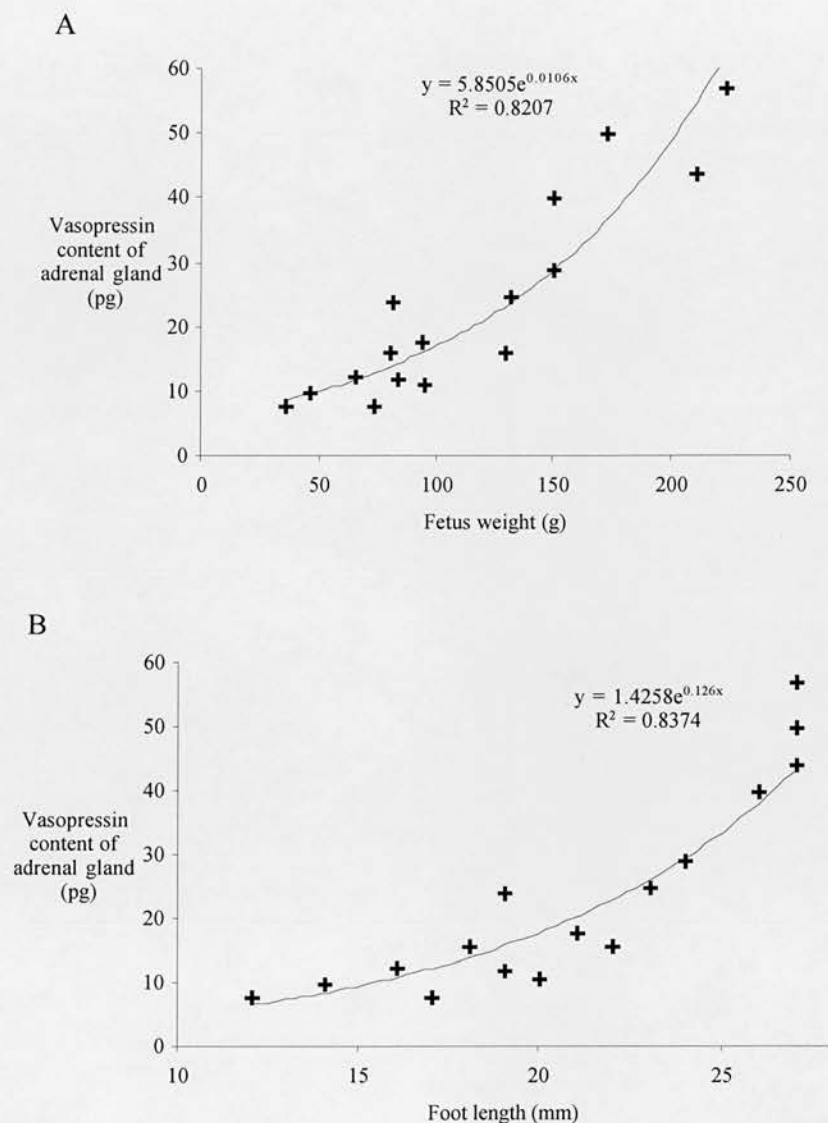


FIGURE 5.4 Vasopressin content of acetic acid extracted human foetal adrenal glands

Adrenal glands from foetuses of varying gestation ages were homogenised in 10ml of 1M acetic acid. After centrifugation, the supernatants were extracted using C8-silica cartridges. The resulting eluates were dried down, raised up in assay buffer and assayed using the *s278* antibody. The resulting values for the total adrenal content are plotted against foetus weight (A) and foot length (B) to demonstrate the change in content of the gland with development.

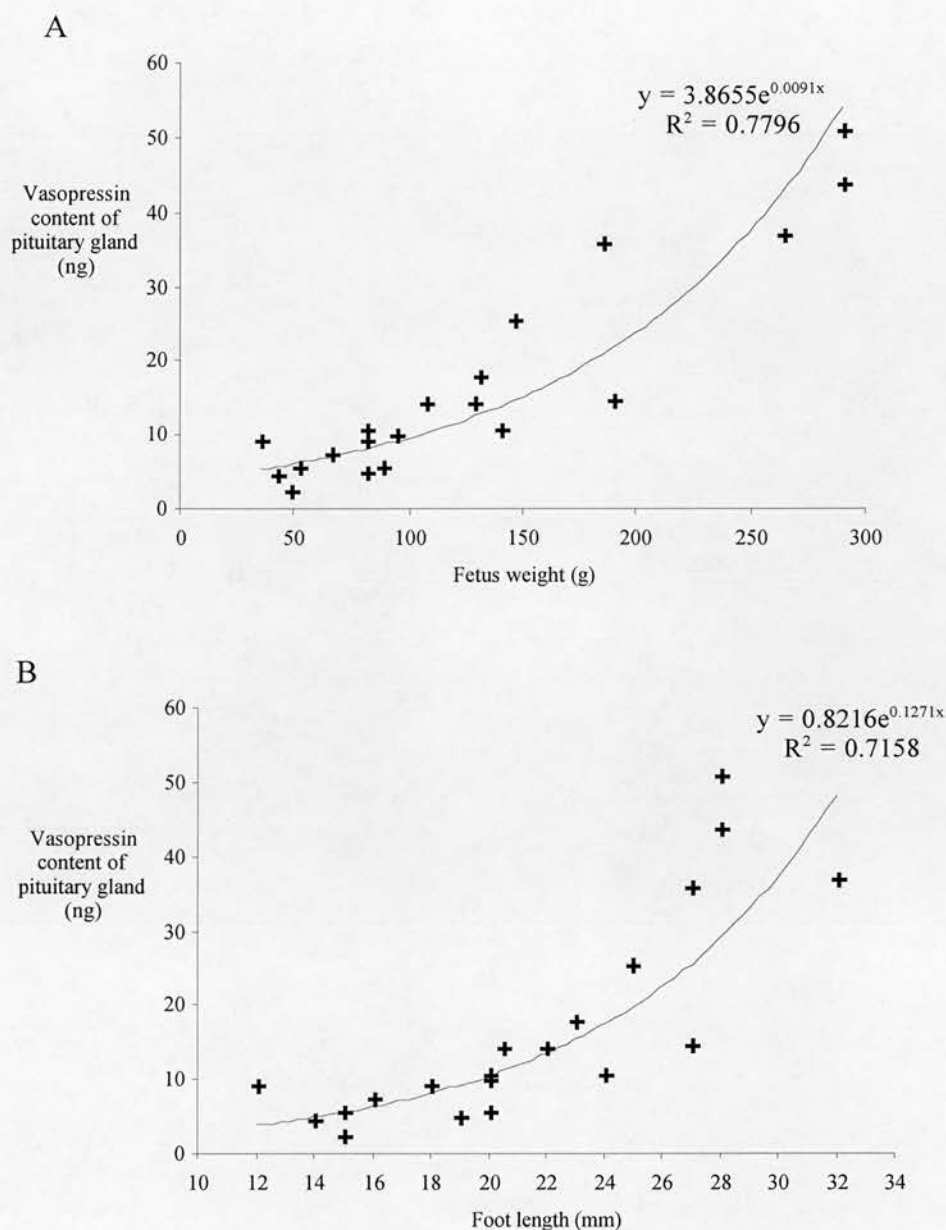
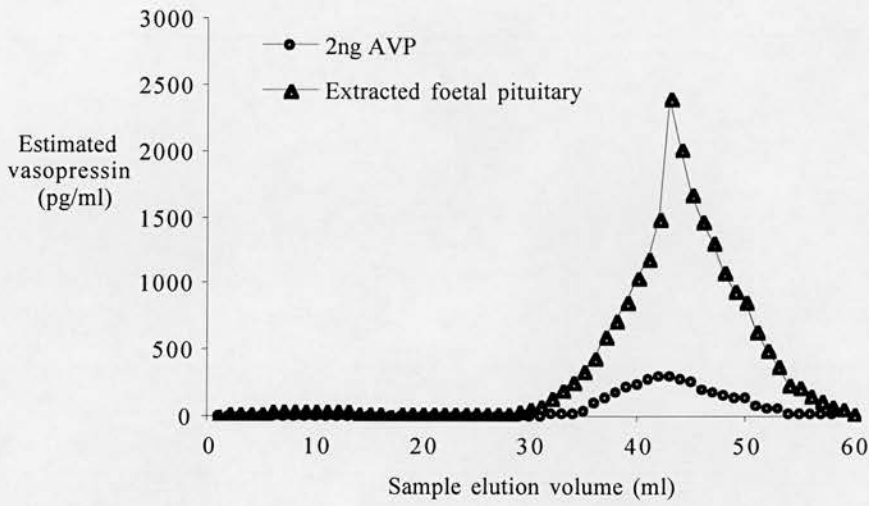


FIGURE 5.5 Vasopressin content of acetic acid extracted human foetal posterior pituitary glands

The entire dissected middle cranial fossa including the posterior pituitary glands from foetuses of varying gestation ages were homogenised in 1M acetic acid. After centrifugation, the supernatants were extracted using C8-silica cartridges. The resulting eluates were dried down, raised up in assay buffer and assayed using the *s278* antibody. The resulting values for the total gland content are plotted against foetus weight (A) and foot length (B) to demonstrate the change in content of the gland with development.

A



B

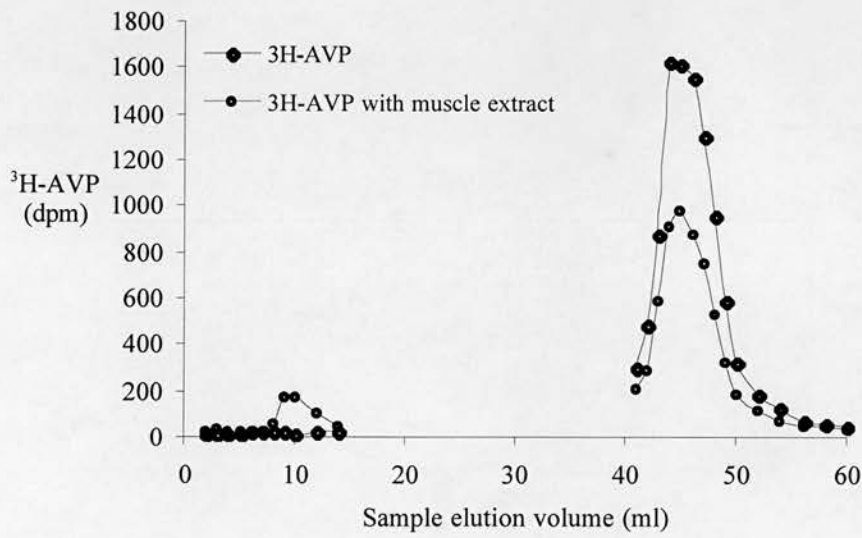


FIGURE 5.6 Standardisation of the gel filtration of AVP
(legend overleaf)

FIGURE 5.6 Standardisation of the gel filtration of AVP

A: In order to standardise the elution volume of AVP, standard vasopressin was eluted using Sephadex G-25. Quantities of synthetic AVP (2ng) in 2 ml aliquots of 1M acetic acid were added to the Sephadex columns. The eluates were collected in fractions of 1 ml and assayed using the *s278* antiserum.

A: A whole pituitary gland from a foetus (Estimated age 19 weeks) was homogenised in 8ml of 1M acetic acid. After centrifugation a 2ml aliquot of the supernatant was applied to the column. Eluted fractions of 1ml were assayed using the *s278* antiserum.

B: ^3H -AVP with an activity equivalent to 2ng was added to 12ml of acetic acid and mixed. A 2ml aliquot was loaded onto the Sephadex G-25 columns. Fractions of 1 ml were collected and aliquots were counted after addition of liquid scintillant.

B: The same volume of acetic acid, containing the same amount of tritiated AVP, was used to homogenise 1g of muscle from a 17 week old foetus. After centrifugation, 2ml of the supernatant was added to the Sephadex column. Fractions of 1ml were collected and mixed with liquid scintillant and counted for beta radiation activity.

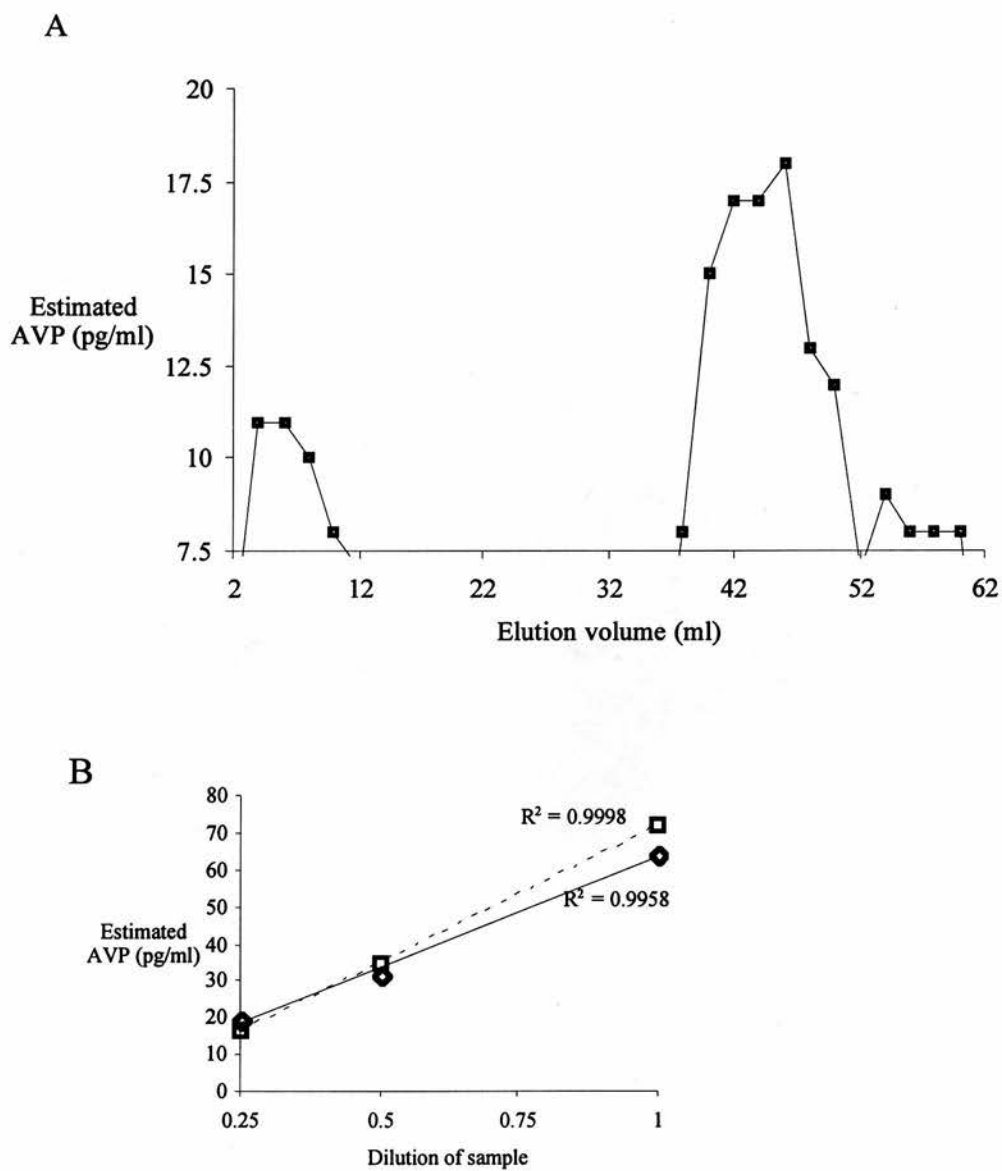


Figure 5.7 **Elution of immunoreactivity from extracted foetal adrenal gland**
(legend overleaf)

Figure 5.7 Elution of immunoreactivity from extracted foetal adrenal gland

A: Foetal human adrenal glands (no gestation ages were noted) with a combined weight of 3g were homogenised in 10 ml of 1M acetic acid. After centrifugation, 2ml of the aliquot was loaded onto the Sephadex G-25 column and eluted. Fractions of 1ml were collected. Every other eluted fraction of 1ml was assayed using the *s278* antiserum.

B: The alternate tubes from the immunoreactivity around the void volume were pooled and C8-silica extracted. The same was done with fractions leading up to the AVP associated peak (odd numbered fraction volume from 39 to 45), and fractions on the down slope of this peak (fractions 47 to 53). The resulting concentrated samples were raised up in assay buffer, filtered using 10kDa cutoff filters, and the filtrates assayed using the *s278* antibody assay. The measurement from the concentrated sample from the void volume fractions was below the lowest detectable limit, but the samples from the peak had immunoreactivity demonstrating parallel dilution with AVP standard, shown here.

Elution volume (ml)	Sample before filtering		Sample after filtering	
	No dilution	1/2 dilution	No dilution	1/2 dilution
0-10	685	22	N/E	N/E
11-20	1217	N/E	7.7	N/E
21-30	655	11.5	N/E	N/E
31-40	355	16.4	N/E	N/E
41-50	291	16.5	19.3	11.7
51-60	55.6	13	14.6	9.7
61-70	21.8	11.9	N/E	N/E

Figure 5.8 Identification of AVP-like immunoreactivity in foetal human skeletal muscle

Foetal muscle samples that, according to assays summarised in Figure 5.3, contained higher amounts of AVP were identified and duplicate homogenisations and C8-silica extractions carried out. The resulting samples were dissolved into small volumes of hot 1M acetic acid, combined, and loaded onto a Sephadex G-25 column as a 2ml sample. A low but significant level of immunoreactivity was observed in *ALL* fractions, until two column volumes had passed through the column (data not shown). This immunoreactivity did not exceed 17pg/ml in any of the fractions. Accordingly, the remainder of the samples were re-extracted in groups of ten fractions through separate C8-silica cartridges. An aliquot of 150µl was removed for assay, and the remaining solutions were filtered using a 10kDa molecular weight cut off filter, and the filtrate assayed for vasopressin. A very small level of AVP was seen at both the void volume and the approximate volume at which AVP would be expected to be eluted to appear in from the standardisation experiments.

N/E: No Estimate – immunoreactivity was below the lowest standard on the AVP radioimmunoassay.

5.3 Discussion

5.3.1 Posterior pituitary glands

The experiments described in this chapter were carried out to assess the potential of using a C8-silica solid phase extraction step in the quantification of vasopressin in tissues by radioimmunoassay, and so to demonstrate the presence or otherwise of immunoreactivity due to authentic vasopressin in foetal human skeletal muscle. The earlier aim was achieved by showing that synthetic vasopressin could be reproducibly separated from 1M acetic acid contamination at concentrations encountered in physiologically relevant concentrations. The C8-silica cartridges showed no evidence of saturation even when used to isolate as much as 10ng of synthetic vasopressin. When foetal pituitary glands were subjected to homogenisation followed by C8-silica cartridge extraction, measured levels of vasopressin were consistent with those reported elsewhere. Data summarised in Figure 5.5 reflect an increase in the vasopressin content of pituitary glands correlated with increases of foot length and foetal weight. Schubert reports a range of 2ng in foetuses aged 15 weeks to 78ng in foetuses aged 19 weeks (22). Burford and co-workers report of 1ng at 11 weeks gestation to 1000ng at 28 weeks gestation (201). Figure 5.5 represents extractions of pituitary glands from foetuses of 14 to 19 weeks gestation age. The range of estimated vasopressin in experiments performed above was 5ng to 51ng per gland. The samples for this study were dissected and frozen between 1 and 2 hours after expulsion. Schubert and co-workers obtained foetal pituitary samples, which were frozen on dry ice immediately after foetal expulsion for their study, and as such undoubtedly reflect more accurately the content of developing foetal posterior pituitary glands. Nonetheless, the similarity of the data

establishes the validity of the use of C8-silica cartridge extraction for the isolation of vasopressin from highly concentrated samples in 1M acetic acid.

5.3.2 Skeletal muscle

The C8-silica extraction of exogenous vasopressin from samples containing components of muscle showed a lower recovery of peptide, and this reduction in recovery was related to the amount of muscle to volume ratio used for homogenisation. One explanation for this finding is that the high concentration of protein in the extract interferes with the interaction of the peptide and the resin, causing a lower retained fraction of peptide. However, the recovery was still of a sufficiently high level to justify proceeding without modification of the extraction beyond controlling the volume of acid used in homogenisation and the weights of foetal muscle extraction, so that recovery would be consistent for all extractions.

Immunoreactivity was present in many foetal muscle samples, to the extent that it affected the recovery efficiency of low doses of exogenous AVP, but this immunoreactivity was demonstrated to be interference in the radioimmunoassay due to the presence of a substance of higher molecular weight than vasopressin. Even when samples were concentrated to a high degree (more than twenty times), little immunoreactivity that either eluted at the volume of free synthetic or pituitary vasopressin or diluted in parallel with synthetic vasopressin was observed. The possibility that more of the vasopressin is retained in the high molecular weight fraction of the filtration cannot be wholly discounted. Exogenous ^3H -AVP was found in this fraction when introduced to the post-homogenisation supernatant. However, in all cases where this retention of vasopressin was observed, it was accompanied with

a much larger amount of vasopressin immunoreactivity (or radioactivity) at the volume associated with synthetic vasopressin and vasopressin immunoreactivity from pituitary glands. This was not seen in the experiments with foetal muscle extract. The occurrence of apparent vasopressin immunoreactivity in foetal muscle extracts in both gel filtrated 1M acetic acid extracts and C8-silica cartridge eluates is presumably therefore due to non-specific interference by other higher molecular weight proteins that are retained on the adsorbent along with the peptides being studied.

The levels of this interference are not significantly related to growth as shown by Figure 5.3, and so cannot be said to be a feature specific to development. The interference may be a reflection of tissue damage or bruising produced through the process of termination, as it seems the most likely interfering protein is haemoglobin. Homogenates and supernatant are brown in colour, and some of this colour is retained on the column when muscle extract is passed through the column. Upon elution of the retained fraction, when the eluate is dried under vacuum, a brown pellet is formed. The addition of assay buffer causes all of the extracted vasopressin to be released into the buffer, but obviously some of the contaminating protein also dissolves, causing the observed interference, although without any observable discolouration of the sample. This is manifested as an inappropriately large estimated concentration in the affected concentrated extract, which disappears with minimal dilution.

Such interference was not seen in extracts of adult rabbit muscle, which was not largely damaged in sampling, visibly contains less blood, and was observed to produce a much smaller pellet than of foetal muscle when comparable weights of

muscle were extracted. It is also apparent that this interference is not present when extracting blood plasma or serum, when red blood cells are deliberately removed prior to vasopressin extraction, or when lower weights of adrenal gland tissue are extracted. But the observation of this background in the assay reveals an important factor when describing limits of an assay. The calculated lowest detectable concentration of the s278 assay is quoted as being 7.8pg/ml. However, this is an artificial quality of the assay as concentrations lower than the background levels seen in extracts muscle will not dilute normally. The minimal detection limit of the assay is therefore higher when assaying biological samples, and it is a failing of these studies on foetal muscle that this different limit was not found. The limitations of his assay bring in to doubt the reported levels of AVP in Figure 5.4.

The possibility that the high molecular weight interference is due to binding of vasopressin by neurophysin cannot be wholly ruled out. However, the neutral pH of the eluting buffer is not conducive to the stable binding of vasopressin to the neurophysin (256). No assay for neurophysin was available to test this possibility.

Another possibility is that of the presence of a vasopressin degrading enzyme, similar to that observed in extracts of testis (139). The enzyme broke down the tracer and caused a lower radioactive count, which was interpreted as displacement of binding. This seems unlikely for two reasons. First, the muscle was homogenised and boiled in acetic acid, whereas the extraction of the rat took place on ice, perhaps preserving activity that is lost (or simply not present) in higher temperature extraction. Second, the retention of tritiated AVP in the high molecular weight fraction of muscle samples suggests that immunoreactivity is physically retained, whereas the immunoreactivity found in the rats testis at high molecular weight was

found to be caused by to degradation of the tracer in sample tubes in the radioimmunoassay.

Finally, the identity of a specific high molecular weight species capable of binding vasopressin also could include a receptor to the peptide. This would be consistent with the observations of a V1-type receptor in the L6 rat myoblast cell line by Nervi *et al* (195), and would fit with a proposed model of the participation of vasopressin (or vasopressin-like molecule that is recognised by the V1-type receptor) in the maturation of skeletal muscle. Further study to attempt to demonstrate the presence of the receptor was indicated.

The amounts of vasopressin shown in Figure 5.8 are values obtained by pooling more than 20g of muscle, and are therefore the equivalent of no more than 2.5pg for every gram of muscle tissue extracted. This explains why it was impossible to detect vasopressin immunoreactivity, either as a peak from Sephadex G-25 chromatography or in assays where non-specific interference was observed from extractions of foetal muscle from mid-trimester fetuses. This is contrary to later findings of Smith *et al* (187), but is in agreement with an earlier observation of Smith that no significant amount of vasopressin (or oxytocin and vasotocin) could be demonstrated in muscle sampled from 10 to 24 week old fetuses (257). Vasopressin could not be demonstrated in either embryonic or foetal rat muscle by Adamo (258). The proposed model of vasopressin action in foetal muscle requires the generation of a local concentration of vasopressin as high as that required to exert an effect *in vitro*. The lowest concentration that was observed to stimulate an increase in fusion of L6 myoblasts was $1 \times 10^{-9} \text{M}$, or in the range of 1ng/ml (194, 195). For 2.5 pg/g of vasopressin to create a concentration of 1ng/ml within a g of tissue would need the

vasopressin to be contained within 2.5 μ l of interstitial fluid which, assuming the density of foetal muscle is similar to adrenal tissue, is the equivalent of 0.25% of the total volume of a gram of muscle. Nussdorfer quotes a figure of 2-3% for the percentage volume of adrenal tissue (180), and although adult skeletal muscle is densely packed and may conceivably have a density approaching that, foetal muscle is comprised of many much smaller unfused cells with a far lower density. Thus, the conclusion drawn from the results described above is that a significant amount of vasopressin could not be identified any foetal muscle sampled.

5.3.3 Foetal adrenal glands

It was also possible to demonstrate low amounts of vasopressin in foetal human adrenal glands. This immunoreactivity displaced tracer binding over a range of dilutions in the RIA for vasopressin in parallel with the synthetic peptide, and when present in sufficiently high levels was shown to elute at the same volume as vasopressin from a pituitary gland when subjected to gel filtration (Figure 5.7). The relationship found between the development of the foetus and the amount of vasopressin immunoreactivity in the adrenal gland extract is one that is considered to merit further investigation. Levels found in those glands from smaller fetuses are low, and though it is conceivable that these levels are due to the same contamination as is seen in foetal muscle, the concentration of the immunoreactivity and the mobility of this immunoreactivity when concentrated C8-silica extract from pooled adrenal glands subjected to G-25 chromatography suggests otherwise. It is unfortunate that adrenal gland weights were not noted at the times of extraction, but it seems obvious that the weight of the adrenal glands increases with gestation age

and therefore body weight and foot length. Subsequent sampled adrenals were weighed, and the range of weights of the glands were between 0.3g and 1.6g for estimated gestation ages 14 weeks to 19 weeks. Taking these as a rough guide for estimating the peptide content per gram of tissue (the estimated peptide contents found in the extracted adrenals ranged from 8pg to 57pg), the data compare closely with one of the two reports of vasopressin concentration in the foetal human adrenal gland. Nicholson and co-workers reported a level of 80pg/g from adrenal tissue pooled from five fetuses of differing gestation ages (138). Neither the ages nor the weight of the adrenal glands that produced this figure are stated. It should be noted that the vasopressin per gram of tissue ratio is lower in larger adrenal gland samples.

Ravid and co-workers detected immunoreactivity in the adrenal glands of 9 foetal or neonatal samples from fetuses ranging from 28 to 40 weeks gestation, and levels were close to those described here (ranging from 2.8 to 59pg/gland) (164). However, some of the fetuses were anencephalic, and the time of delay between death and freezing prior to extraction ranged from 5 to more than 48 hours, and given these factors it is difficult to assess the usefulness of the information provided by this study. They also show extremely low amounts of immunoreactivity gained from rat foetal adrenal glands. The reason for this could be the low yield from the Vycor glass powder extraction, but the extraction efficiency for this method has been shown to be better than 80% (259). The foetal rat adrenal glands that were extracted were extremely small (less than 1mg), and so it is not unexpected that low amounts are detected.

mRNA for vasopressin has been detected in adult rat adrenal tissue (260). Along with the observations of Nicholson and co-workers that the neurophysin could

also be found in foetal human adrenal glands (138), the results shown in Figure 5.4 suggest a developmental aspect of the ontogeny of adrenal gland associated vasopressin. However, the conclusions drawn from the study of C8-silica extracted muscle was that extraction with C8-silica sometimes produced samples that demonstrated a high background, and although further studies were carried out to confirm the nature of the immunoreactivity from pooled samples, the veracity of the presented relationship between foetus age and levels of vasopressin in the adrenal remains to be definitively described. The use of molecular weight filters after solid phase extraction would be essential in further work to elucidate this relationship.

Nonetheless, significant concentrations of AVP were observed in foetal adrenal glands. Whether this AVP has a role in the development of the foetal adrenal gland or some aspect of physiological control in the foetus remains to be seen, and further study to localise the vasopressin immunoreactivity at a cellular level was indicated.

Chapter 6

Immunochemical Analysis of Extrahypothalamic AVP and the V1a-Vasopressin Receptor

6.1 Introduction

The results of experiments described in Chapter 5 hint at the presence of a vasopressin binding factor in extracts of foetal skeletal muscle. Vasopressin immunoreactivity was retained in high molecular weight fractions when extracts of tissues were subjected to Sephadex G-25 gel filtration, suggesting the binding, specific or otherwise, of vasopressin.

The presence of a receptor on the myoblast cell is a requirement of any model describing a role for vasopressin in the development of mature muscle, as a means of transduction of the vasopressin signal to initiate fusion. In the rat L6 myoblast cell line, functional vasopressin receptors have been identified, and characterised as the V1a subtype (191, 194). Demonstration of presence of the vasopressin receptor, and localisation of the receptor at a cellular level in a developing muscle would be important.

6.1.1 Immunoblotting

It was decided to use an established method to first try to demonstrate the presence of the vasopressin receptor in foetal skeletal muscle tissue.

Gel electrophoresis of proteins is a powerful technique, because it allows the separation and resolution of the protein components of a sample. Analysis of proteins

by staining using substrates such as Coomassie dyes allows the examination of the overall pattern of proteins within a sample, but specific identification of a protein requires immunochemical detection, and gels used in electrophoresis are too fragile to tolerate the procedure.

Blotting - the transfer of separated proteins onto a membrane - immobilises these proteins and makes them more accessible to sensitive methods of detection that allow identification. The technique originated from capillary blotting of DNA as described by Southern (261). Towbin used electroelution to transfer proteins from gel to membrane (262), but it was Burnette who first coined the term "Western blotting" in describing the transfer of proteins from an SDS-polyacrylamide gel to a nitrocellulose membrane with the subsequent antibody based detection of a protein on the membrane (263). The technique has developed to the point now where it is a highly effective way to use antibodies to detect the presence, molecular weight, and relative abundance of proteins from samples. The technique has been further enhanced with the advent of chemiluminescence (the emission of light from a chemical reaction), a non-hazardous alternative to radiography that requires no special facilities or disposal, and is flexible in terms of adjustment of conditions and exposure to film to generate images. It also allows the investigator the opportunity to re-examine the same blot with different antibodies to profile a sample.

Few reports exist of the use of Western blotting to characterise the V1a receptor. This is undoubtedly due to the low level of expression of the receptor even in tissues where it is shown to exert an effect (41). The V1a receptor is usually characterised in other ways, such as radio-ligand binding studies or the use of non-peptide ligands (56). Park and colleagues demonstrate the presence of the V1a

receptor in the renal medulla using a variety of techniques including Western blotting, and characterise the receptor as a 44.2kDa protein (58).

Use of the antibody *vivian* has been reported in positive Western blot analysis of cultured cells (221), and this antibody was used to attempt to identify the receptor in foetal extracts.

6.1.2 Immunohistochemistry

Immunoreactivity with a vasopressin antibody was observed in samples of extracts of foetal human adrenal gland (Chapter 5). Results describing immunoreactivity in the rat adrenal medulla would confirm the effectiveness of the technique, as this has been previously reported (164, 166), and localisation of the vasopressin in foetal adrenal tissues would give an indication as to the role, if any, of vasopressin in the developing foetal adrenal gland. The extraction of vasopressin from skeletal muscle could be further tested by the use of an alternative antibody raised against a vasopressin antigen. It was decided to utilise immunohistochemistry to attempt to localise vasopressin and vasopressin receptor immunoreactivity.

Immunohistochemistry uses an antibody to link a cellular antigen specifically to a stain, implying that any specific staining at a location is due to the presence of that antigen. Tissues sections can be prepared either from frozen samples, where the tissue is mounted at low temperature (usually after snap freezing) and sections are cut in a cryostat (essentially a refrigerated microtome), or from paraffin wax embedded blocks where the tissues has been fixed, usually by an aldehyde based or an alcohol based fixative. Frozen sections have an advantage in that they display good preservation of antigen immunoreactivity, but do not allow fine resolution of

morphological detail. Fixation by its nature involves formation of chemically stable links to stabilise the morphology of the tissue, and loss of antigen immunoreactivity is a common effect most fixation methods. However, antigens can be 'retrieved' by the breaking of these crosslinks, and significant loss of immunoreactivity can be minimised after appropriate treatment.

For the purpose of localisation of vasopressin and the associated V1a subtype receptor, it was decided to use sections prepared from samples that had been fixed in formalin, processed, and embedded in paraffin wax. Use of paraffin wax embedded sections also has the advantage that readily available archive material can be accessed once a method has been established, allowing greater numbers of samples to be processed.

6.2 Results

6.2.1 Analysis of Western blots

Two extraction methods were used identify the vasopressin, and two methods were used to try to elucidate the relative levels of expression at different stages of gestation.

Samples extracted using TRIzol reagent demonstrated that the vasopressin receptor could be positively identified in samples from 15 weeks gestation and older (Figure 6.1A), and that the molecular weight of the positive signal was consistent with both an internal control (Figure 6.1A lane 3) and published reports (58, 221). V1a was also identified in a foetal liver sample, although signal was only seen at the higher molecular weight associated with V1a. In the blot shown, signal emanating from the higher molecular weight region was blocked, presumably by the high

amount of protein transferred at that molecular weight from a gel already overloaded with protein in order to generate an image. Subsequently it was realised that the levels of protein, although ostensibly similar, showed variation in their composition. Levels of haemoglobin in the homogenate, while undoubtedly the major component of protein, varied markedly between samples, and so normalising using protein concentration involved inherent errors.

In an attempt to gain information about the developmental expression of the vasopressin receptor, the amount of protein in lysis buffer extracted samples added to a lane was altered with reference to a positive control for the development of skeletal muscle, dystroglycan β – dystroglycan (224). Positive signal for this muscle specific marker was seen in all foetal muscle samples (Figure 6.1). No signal was seen in the positive control sample, human liver.

Positive signal was seen in muscle samples of older foetuses, but it was not possible to gain definitive identification of the receptor at any age. Figure 6.2 shows a blot which typifies this. The positive control sample (lane 1) gave a single band at approximately 43kDa. This is in agreement with data shown by Park *et al*, which show a positive band at approximately 44.2kDa (58). However, muscle samples gave multiple strong bands, with only a weak band at the expected molecular weight. (Whilst negative control experiments where *vivian* was replaced with normal rabbit serum did not show a similar pattern at the predicted molecular weight of the V1a-vasopressin receptor, signal *was* seen at higher molecular weights (data not shown).) This is in agreement with a pattern reported by North *et al* (221). North explains the pattern of banding gained with *vivian* by citing reports that the receptor weight is much higher with glycosylation of the protein (57). The interpretation of the blot

shown in Figure 6.2 is that a positive identification of the V1a receptor has been made in at least the sample of muscle from the 18 week foetus. However, it was not possible to make a quantitative statement about the relative abundance of β -dystroglycan and V1a receptor in the samples.

6.2.3 Immunohistochemical analysis

6.2.3.1 *Brain tissue*

Control tissue stained positively for vasopressin, in keeping with the observation that the hypothalamus is the major site of vasopressin synthesis, and the posterior pituitary gland is the major site of storage and release of vasopressin. Absence of staining with the negative control serum (Figure 6.3A), which was generally observed in all negative control slides, is replaced by a strong specific stain when slides were incubated with *gonzo* (Figure 6.3B). High magnification reveals the staining to be entirely cytoplasmic and entirely within structures identifiable as neuronal in nature (Figure 6.3C). Also shown is the positive staining seen in the posterior pituitary glands from a foetus of 14 weeks estimated gestation age (Figure 6.3D).

6.2.3.2 *Muscle tissue*

Hemotoxylin and eosin stained slides confirmed that muscle was maturing over the middle trimester. Muscle taken from early foetuses generally showed much less differentiation of the myoblasts, which were more numerous. Myoblasts are distinct from mature muscle cells by their mononuclear nature. The nucleus is

usually centrally positioned. This can be seen in Figure 6.4A. The muscle from a 14 weeks foetus is distinctly unlike mature skeletal muscle. Muscle cells in an 18 week old foetus are generally much more dense and nuclei are peripherally positioned on those cells that have fused and are entering a phase of growth and further fusion (Figure 6.4B). However, there are still myoblasts present in the tissue. These observations are qualitative in nature, and no attempt was made to standardise the appearance of a typical sample and correlate this with weight or foot length.

Positive staining was seen with the use of a commercial antibody to vimentin (Figure 6.5) The strong staining seen in younger samples was reduced with older samples, but this is again a qualitative judgement. This is in agreement with observations by Sarnat and co-workers, that foetal skeletal muscle expresses vimentin at all gestation ages (225) and demonstrates that that the tissue was properly fixed and antigen retrieval was properly performed. Absence of, or weak staining is therefore due to a lack of the antigen in the tissue examined rather than the antigen being inaccessible to the antibody.

It was not possible to achieve positive staining with either the antibody to AVP or the vasopressin V1a receptor. Increasing the concentration of the antibody only increased the background without any appreciable specific staining. Staining gained with Vivian was light, and not obviously different from the background staining associated with *gonzo*.

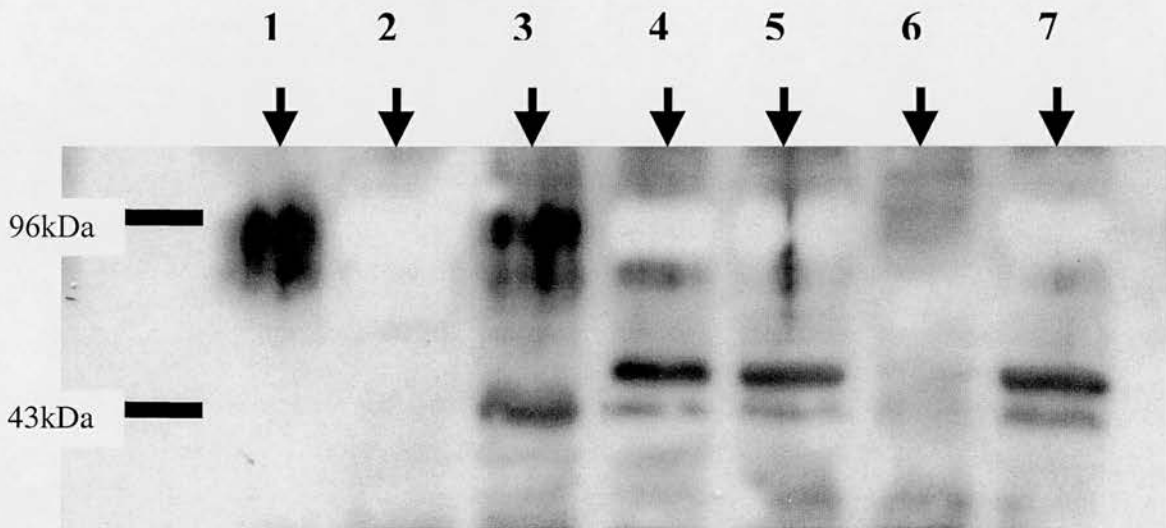
6.2.3.3 Adrenal glands

Foetal human adrenal glands also showed a progression of development associated with increased gestation age in the samples that were stained with

haemotoxylin and eosin. The permanent cortex lining the gland generally was seen to thicken as gestation age increased. No significant staining could be found in foetal adrenal medulla cells of any of the samples examined, although it was never sought to identify foetal medulla cells.

The phenomenon of the incursion of medullary tissue into adrenal cortex in rat adrenal glands is shown in Figure 6.7. The classical scenario of the distinct layers of the gland shown in Figure 6.7A is in contrast to the often seen more intimate situation. The higher power photographs clearly show the difference between the cell types of the island medullary cells and the surrounding cortex cells. Positive staining was repeatedly observed in rat adrenal medulla tissue. Examples of the medullary rays and islands of chromaffin cells in the cortex, that were described by Gallo-Payet and colleagues (181), are shown in Figures 6.8B and C. Significant staining was observed in the rat medulla, confirming previously published observations (166). Staining was most intense in cells that were intercalated within the cortex tissue - this was observed regardless of the location of the cells within the cortex.

A: Blot probed with *vivian* antibody



B: Blot probed with anti- β -dystroglycan antibody

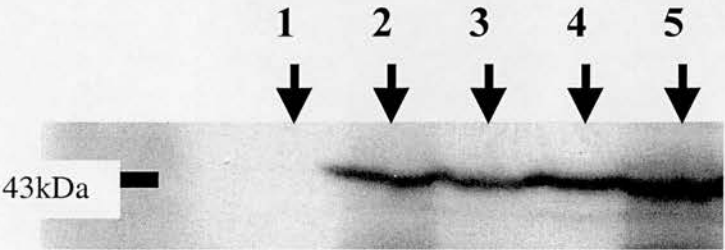


FIGURE 6.1 Normalisation of foetal muscle samples for the Western analysis of vasopressin receptor
(legend overleaf)

FIGURE 6.1 Normalisation of foetal muscle samples for the Western analysis of vasopressin receptor

A Protein samples of foetal muscle were normalised by absorbance at 280nm and equal amounts of reduced protein were loaded into each lane. Shown is a blot where sufficient volume was added to generate positive signal in some of these samples. Foetal liver extract and protein extract of MCF-7 cells were added as positive controls.

Samples

- 1: Foetal liver
- 2: Empty lane
- 3: MCF-7 cell extract
- 4: 18 week old foetus
- 5: 16 week old foetus
- 6: 13 week old foetus
- 7: 15week old foetus

B Extracts produced by homogenisation in lysis buffer of samples of foetal muscle of various gestation ages were assayed for protein content. 25µg of reduced protein was loaded in each lane and separated by SDS-PAGE using a 12% polyacrylamide gel. The separated proteins were transferred to a membrane and incubated with an anti-β-dystroglycan antibody overnight at 4°C. Developed blots were used to adjust the amount of protein added to give a qualitative rising concentration of positive signal, in an attempt to establish the relative changes in expression of β-dystroglycan and the vasopressin V1a receptor.

The relevant portion of a developed blot is shown along with the position of the migration of the closest molecular weight marker.

Samples

- 1: Human liver positive control
- 2: 12 week old foetus
- 3: 15 week old foetus
- 4: 16 week old foetus
- 5: 18 week old foetus

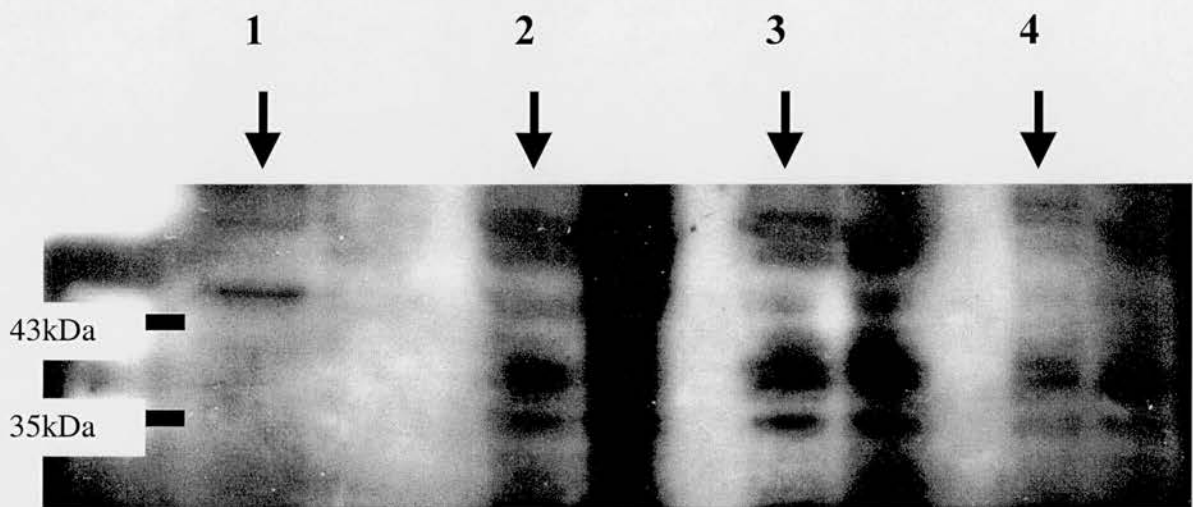
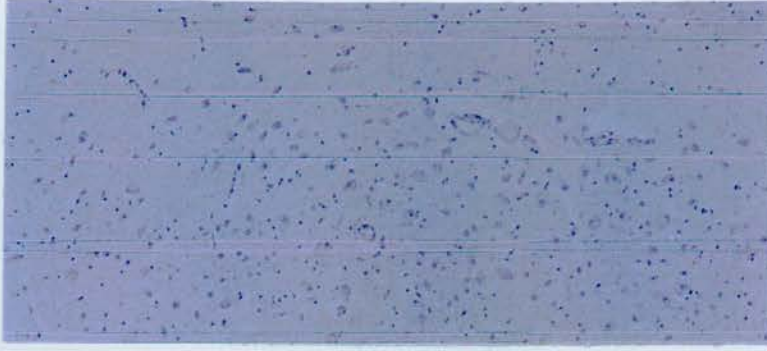


FIGURE 6.2 Western analysis of foetal muscle extracts

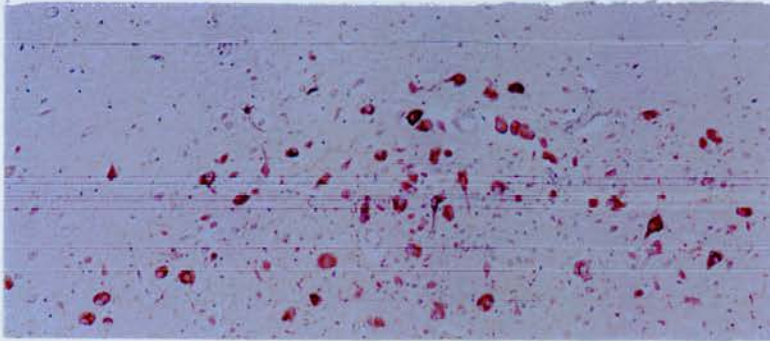
Extracts from samples of foetal muscle, normalised for increasing concentrations of beta-dystroglycan (described in Figure 6.1), were reduced and separated by SDS-PAGE using a 12% polyacrylamide gel. The separated proteins were transferred to a membrane and incubated with *vivian* antibody overnight at 4°C. The relevant portion of the developed blot is shown along with the position of the migration of the closest molecular weight marker.

- 1: Human liver positive control
- 2: 18 week old foetus
- 3: 15 week old foetus
- 4: 12 week old foetus

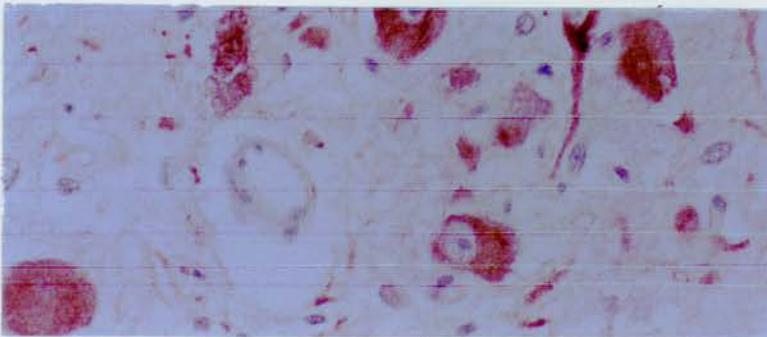
A



B



C



D

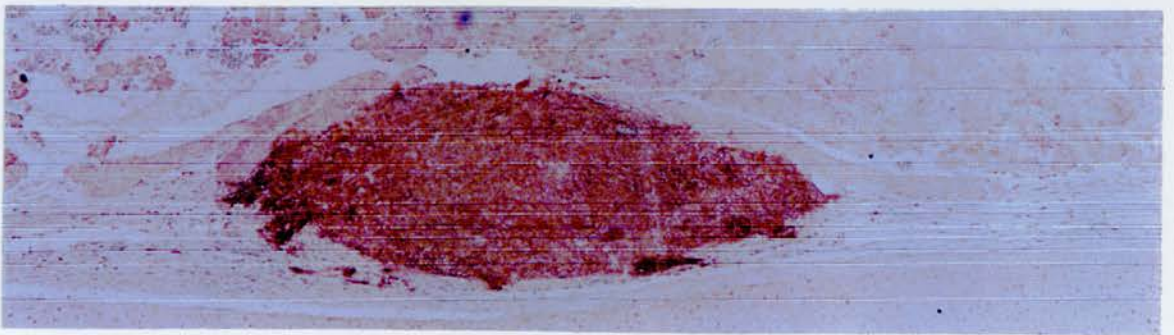


FIGURE 6.3 Positive staining of tissues known to express AVP
(legend overleaf)

FIGURE 6.3 Positive staining of tissues known to express AVP

A Staining was absent when the section is incubated with the negative control serum. This which was generally observed in all negative control slides.

Magnification x40

B Shows a low power view of the positive staining in magnocellular neurones of the hypothalamus. The section was incubated with *gonzo* diluted to 1/400.

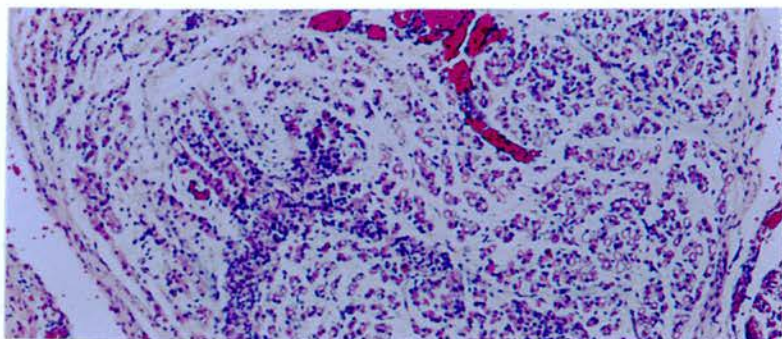
Magnification x40

C A single magnocellular neurone is shown at high power. Staining is confined to the cytoplasm, and the nucleolus is clearly visible at the centre of the nucleus. The section was incubated with *gonzo* diluted to 1/400.

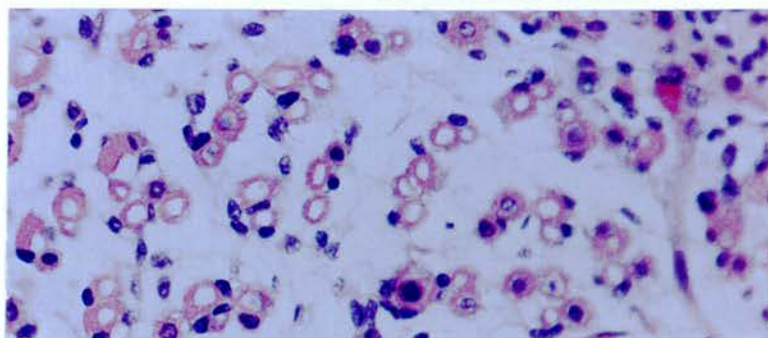
Magnification x200

D The strong positive staining seen in the posterior pituitary glands from a fetus of 14 weeks estimated gestation age. The section was incubated with *gonzo* diluted to 1/400.

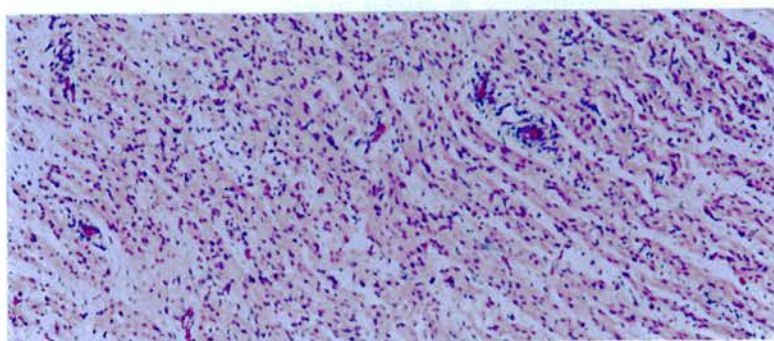
A



B



C



D

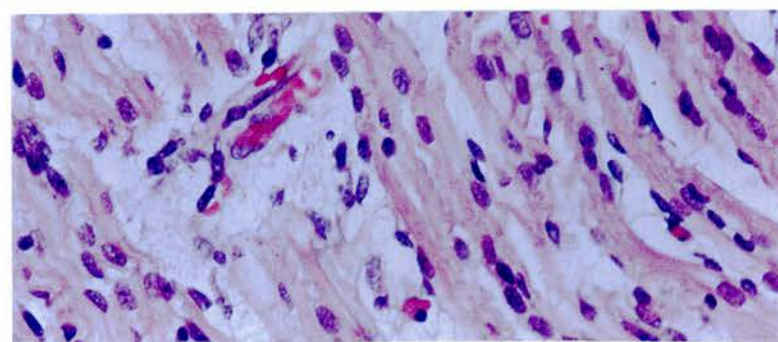


FIGURE 6.4 Morphological changes in developing foetal skeletal muscle
(legend overleaf)

FIGURE 6.4 Morphological changes in developing foetal skeletal muscle

The figures demonstrate the qualitative changes that happen in the development of muscle in as short a period as a month.

Sections of muscle were stained with hemotoxylin and eosin.

A Muscle tissue from a 14 week old foetus is distinctly unlike mature skeletal muscle. The majority of the muscle cells in the tissues were myoblast cells.

Magnification x20

B Myoblasts are distinct from mature muscle cells by their mononuclear nature. The nucleus is centrally positioned in these cells. Many of the cells in this section have lost their nucleus, leading to the pink halo structure seen here.

Magnification x200

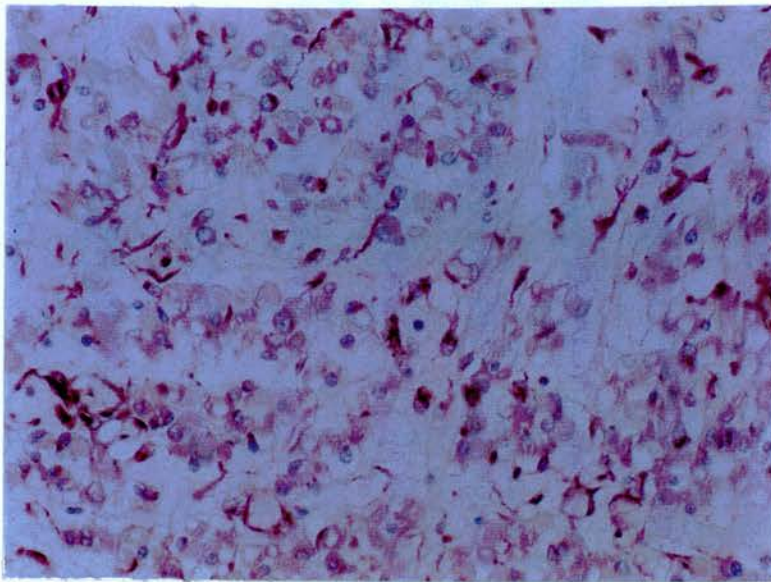
C Muscle tissue taken from an 18 week old foetus is generally much more dense. Fusion of cells leads to the formation of larger myofibrils. Nuclei are peripherally positioned on those cells that have fused. The cells enter a phase of growth and further fusion.

Magnification x20

D More typical morphology associated with skeletal muscle is seen in this longitudinal section of skeletal muscle sample, from a foetus of 18 weeks gestation. Myoblasts have fused to form longer myofibrils, containing multiple peripheral nuclei. Striations of the muscle tissue is sometimes seen. There are, however, still many myoblasts seen in tissues taken from foetuses at this stage in gestation..

Magnification x200

A



B

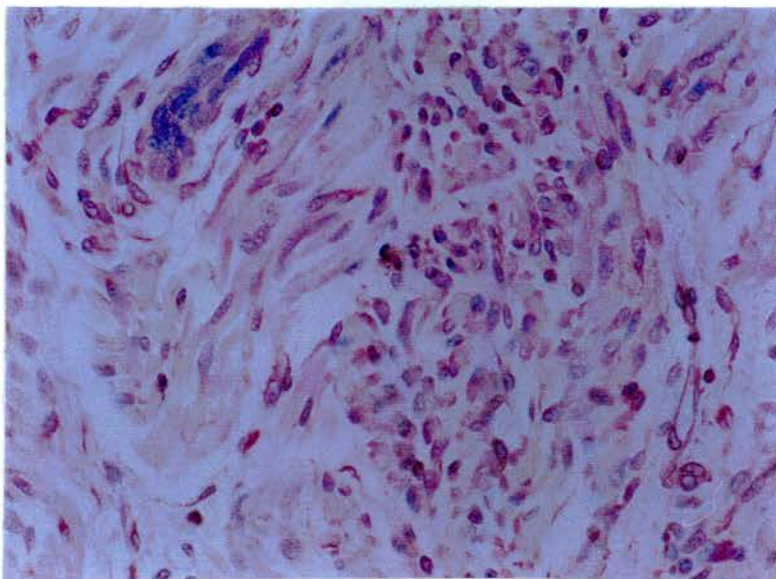


FIGURE 6.5 Positive staining for a marker of muscle development
(legend over leaf)

FIGURE 6.5 Positive staining for a marker of muscle development

The effectiveness of the antigen retrieval process was demonstrated using a commercial antibody to vimentin. Slides were counterstained with hemotoxylin.

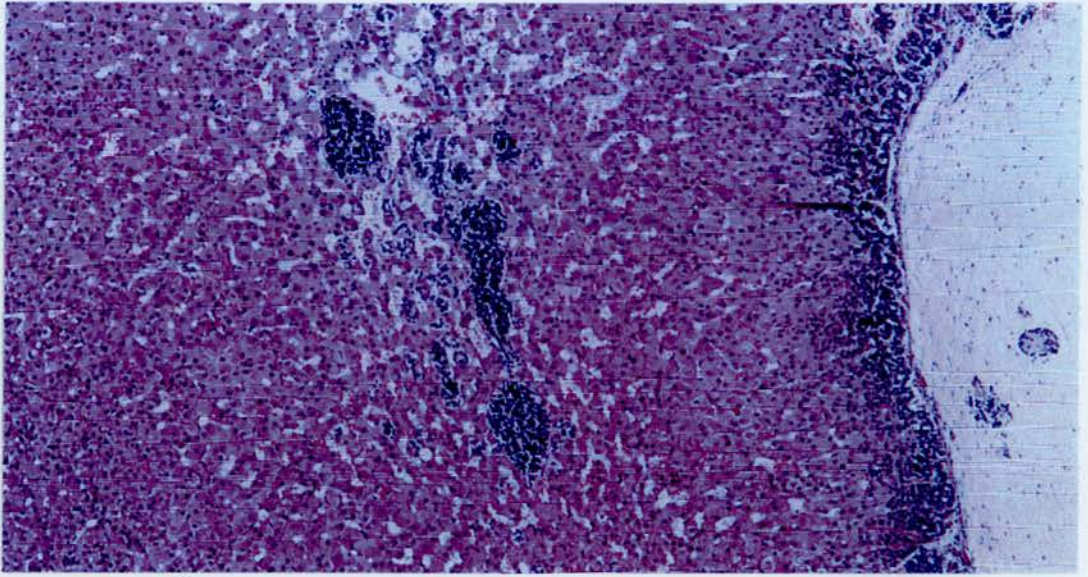
A Positive staining is seen in the myoblast cells of a section from a 14 week old foetus. The staining is non nuclear, in keeping with the description of vimentin as an cytoplasmic intermediate filament.

Magnification x40

B Positive staining is much less dense on a section of tissue which is more developed. Staining is less dense in the myofibril cells seen in this section from an 18 week old foetus.

Magnification x40

A



B

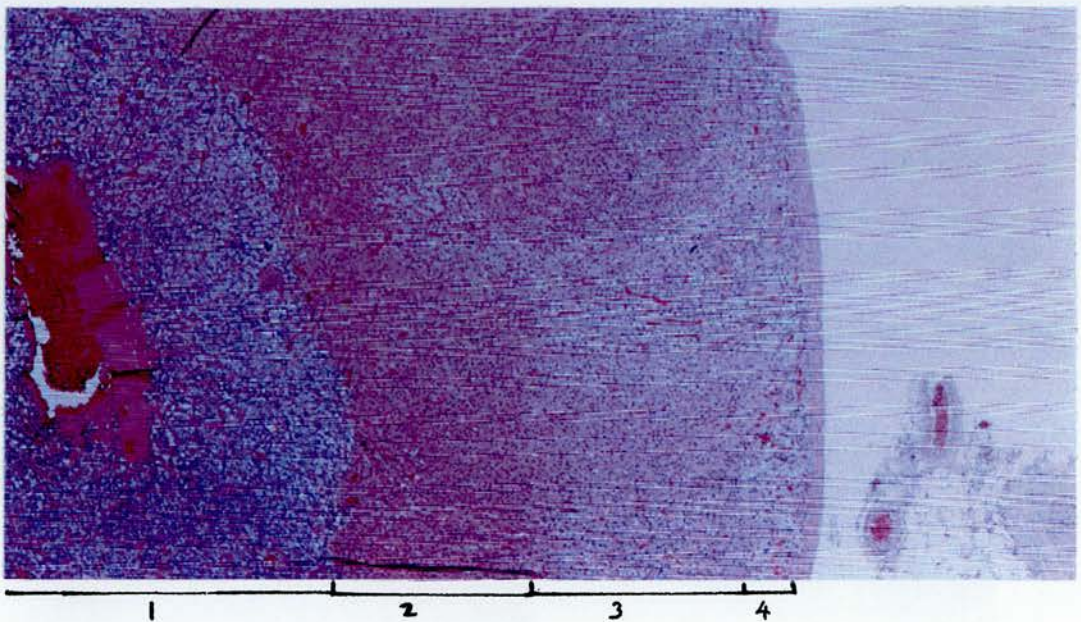


FIGURE 6.6 Development of medulla of adrenal gland
(legend overleaf)

FIGURE 6.6 **Development of medulla of adrenal gland**

A The majority of the cells in the foetal adrenal gland are large eosinophilic cells, referred to as the foetal zone. The other cells seen are permanent cortex cells, seen on the edge of the tissue and at the centre. The cells are basophilic, and have tightly packed nuclei. The gland is arranged in tubes with permanent cortex lying over the foetal zone cells. The foetal adrenal lacks a definitive medulla. Chromaffin cells are present but scattered throughout the cells and difficult to identify in hemotoxylin and eosin sections.

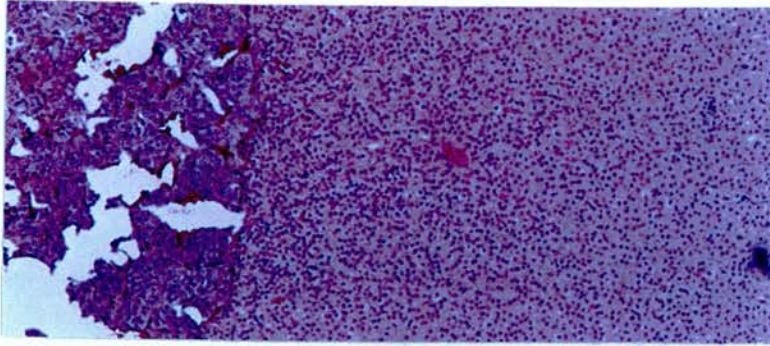
Magnification x20

B At birth, the foetal adrenal gland reduces in size rapidly, the permanent cortex cells undergo growth and maturation to form the familiar three layered structure of the cortex, and with the disappearance of the foetal cells the chromaffin cells aggregate to form the central medulla.

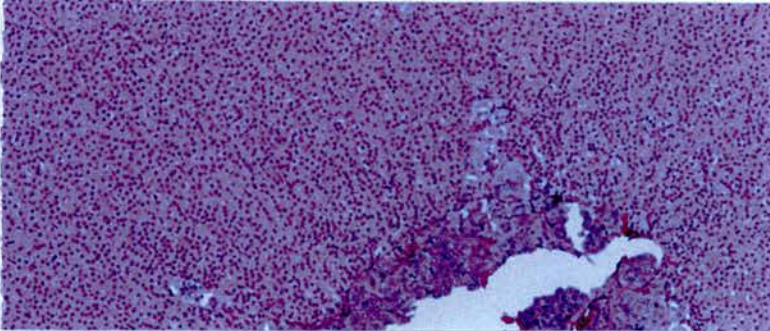
1= medulla 2= zona reticularis 3= zona fasciculata 4= zona glomerulosa

Magnification x10

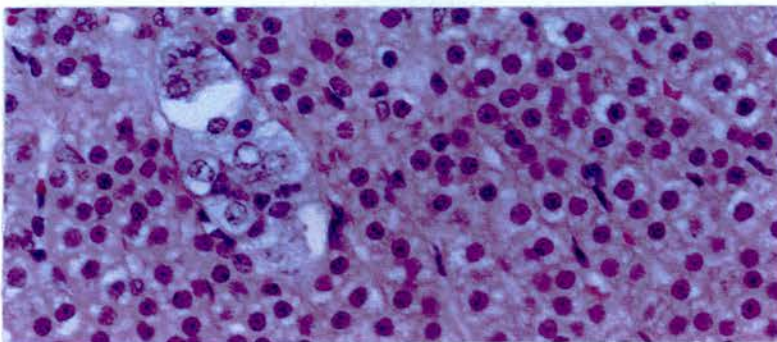
A



B



C



D

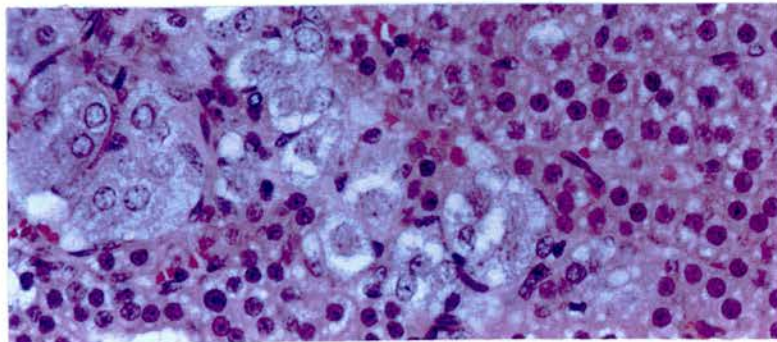


FIGURE 6.7 Rays and islands of medullary tissue in rat adrenal cortex
(legend overleaf)

FIGURE 6.7 Rays and islands of medullary tissue in rat adrenal cortex

The commonly held conception that the medulla and the cortex of the adrenal gland are discrete entities with minimal interaction (Figure 6.7A, Magnification x20) is shown to be false in sections of rat adrenal glands stained with hemotoxylin and eosin.

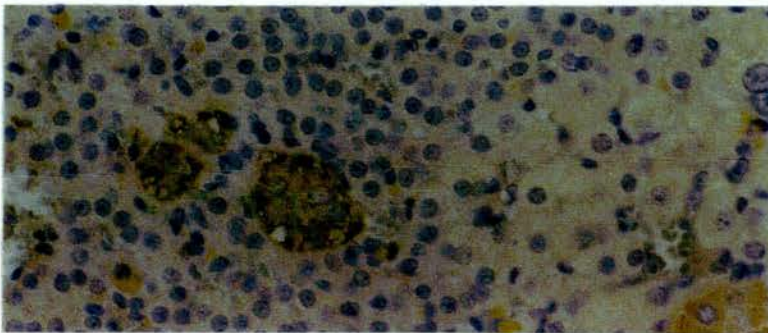
B A ray of blue basophilic medulla cells intrudes into the surrounding cortex, whilst another set of cells are completely surrounded by cortex cells.
Magnification x20

C,D Higher magnification of these two sets of cells definitively shows the morphological difference between the cortex cells and the chromaffin cells.
Magnification x200

A



B



C

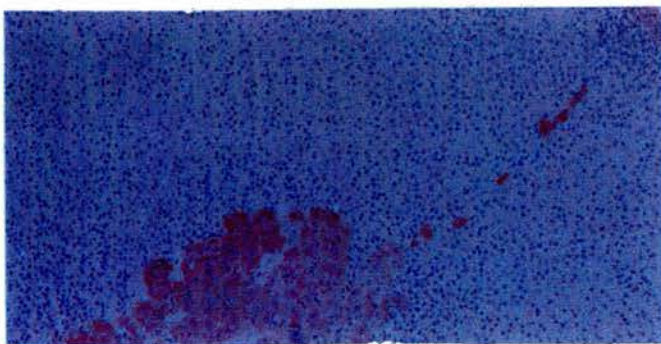


FIGURE 6.8 Positive staining for AVP in rat adrenal glands
(legend overleaf)

FIGURE 6.8 Positive staining for AVP in rat adrenal glands

A Low power view of section of adult rat tissue stained positively for vasopressin using *gonzo* (1/100 dilution). Both a ray of medulla tissue and an island are seen, both with markedly stronger staining than that seen in chromaffin cells more centrally positioned in the medulla.

Magnification x20

B Higher power view of the island of medullary tissue shown in A. The staining is seen to be cytoplasmic and not seen in the nucleus or in surrounding cells of the cortex, indicating it is specific and genuine immunoreactivity.

C Low power view of section of adult rat tissue stained positively for vasopressin using *gonzo* (1/100 dilution). Islands of positively staining medulla cells are seen extending deep into the cortex layers.

Magnification x20

6.3 Discussion

6.3.1 Western analysis

Attempts to define a developmental aspect to the expression of the vasopressin receptor in foetal skeletal muscle were not successful. Younger foetuses did give a positive signal for the V1a receptor, but it was not possible to translate immunoreactivity into a relative abundance of the receptor at different developmental stages, due to the low number of samples and the inherent problems associated with Western analysis of the V1a receptor. The samples used for the initial phase of study were TRIzol extracted and gave rise to a much cleaner sample than those gained from lysis buffer extraction. This is reflected in the variation in the background seen in the two blots probed using the same antiserum preparation (Figure 6.1A and 6.2).

The appearance of the double band of immunoreactivity at the higher molecular weight in Figure 6.1A is consistent with the dimerisation of the receptor, together with differences in the amount of glycosylation of the receptors. No reports of the occurrence or importance of dimerisation of the V1a receptor could be found. Dimerisation of the V2 receptor has been observed, but the interpreted significance of this varies. Sadeghi considers the phenomenon irrelevant to function (or at least not necessary *for* function) (264), whereas Zhu has demonstrated that truncated V2 receptor will inhibit the function of wild type receptors by forming dimers (265). This is complemented by the work of Herbert, who generated SDS-resistant dimers of beta2-adrenergic receptors, and used a peptide portion of the receptor to inhibit the activation of whole receptor molecules (266). The cause of the dimerisation in the samples shown here is not known.

At the molecular weight associated with the single receptor a double band also appears, and whilst this could be explained by the differential glycosylation of the receptor, it would seem more logical to see a smearing of the signal in at both the monomer and dimer level. Deglycosylation of a sample and subsequent Western analysis (similar to studies described by Wheatley *et al* (37)) to detect a shift in the molecular weight of the vasopressin receptor would clarify this.

The presence of the receptor later in gestation rather than earlier would suggest that the receptor appears with increasing maturity of the muscle, rather than any correlation with ability to undergo fusion. The developmental expression of the V1a receptor in liver and kidney tissue has been demonstrated previously (64, 65), but more work is required to define any such situation in foetal muscle.

6.3.2 Immunohistochemistry

Experiments described in this chapter were carried out in order to localise previously described immunoreactivity or the potential for it. Vasopressin had been identified in foetal human adrenal glands and in adult rat adrenal glands through radioimmunoassay. V1a subtype receptors were tentatively demonstrated in a Western blot using extracts of foetal skeletal muscle. Staining was only successfully seen in rat adrenal glands.

The finding that medullary rays stain positively for vasopressin provides an explanation for the observations that cortex tissues contain vasopressin (bovine in particular) (165). When the cortex and medulla are separated by dissection, some medulla is retained within the cortex, giving a positive result when extracted and assayed for vasopressin. These observations also give support to the belief that

vasopressin acts as a paracrine regulator of adrenal cortical function (172). In the absence of a circulatory mechanism to distribute the peptide, and given the relatively low amounts of vasopressin in the adrenal gland itself, it seems logical that the peptide should be distributed in areas where it will be able to exert most effect. This observation may be an artefact of the manner of death of the rats these samples were taken from; there can be few more systematically stressful stimuli than stunning and decapitation, and so the staining may actually reflect the remaining vasopressin that has not been secreted in response to this. Further work is required to clarify this, and will need to involve more sensitive techniques.

Studies to locate receptor immunoreactivity and peptide reactivity in foetal tissue was hampered by the lack of sensitivity of the techniques used. Immunohistochemistry supplied indeterminate results at best, and options to optimise the staining were limited. Western blotting will provide the best method to confirm the developmental appearance of the receptor in muscle tissue. Further attempts to localise the receptor will rely on techniques such as *in situ* hybridisation, as the protein itself is not apparently expressed in sufficient quantities to allow identification by immunohistochemistry.

Chapter 7

Discussion and Recommendations for Further Study

7.1 Summary

The experiments described in this thesis were carried out with the aim of supplying more information about the synthesis of extrahypothalamic vasopressin, and the possible role of vasopressin in the development of skeletal muscle in the foetus. It had been suggested that high concentrations of vasopressin were present in the muscle of human foetuses from approximately 15 weeks to term, with a lowering of the levels correlated with an increase in gestation age. Other studies showed that vasopressin promoted the fusion of cultured cells that retained the myoblast phenotype, and taken together these studies suggest a role for vasopressin in the formation of multinucleated muscle fibres required for the formation of normal muscle.

The occurrence of extrahypothalamic vasopressin is an established fact. Similar studies in the adrenal gland have established a model for the progression of knowledge, from studies based on the immunological profiling of a tissue - the detection of vasopressin and the neurophysin, and associated receptors, to the use of molecular biological techniques to confirm the presence of transcripts of genes for vasopressin and both the V1a and V1b receptor subtypes. It was proposed to carry out an investigation of vasopressin in foetal muscle with a similar pattern. By use of a sensitive and specific radioimmunoassay for vasopressin it was hoped to show that there was a relationship between the amount of vasopressin in the muscle, and the

required amount of myoblast fusion to give rise to sufficient mature multinucleated myofibrils to enter into the growth phase of development at the particular gestation age of the foetus. It was also hoped to localise the immunoreactivity in terms of a cell type or a structure within the developing muscle. As the foetus grows in size, it is assumed that the number of myoblasts diminishes, and so there would be a reduced requirement for high levels of vasopressin.

7.1.1 Initial observations

The use of sheep and rabbit antibodies in the radioimmunoassay for vasopressin was validated. A serum pool to be used as a source of antibody to vasopressin was characterised (*s278* antiserum). It was shown that the antibody was specific and sensitive to vasopressin when compared to other related peptides. A standard curve was established for the *s278* antiserum. When compared with the already characterised assay using a rabbit polyclonal antiserum (*tg1*), the two antisera provided similar extrapolated concentrations of the same sample of synthetic vasopressin in their respective assays.

The assay of extracts of foetal muscle in acetic acid was attempted. Results from the two assays were not similar, and when the samples were diluted did not provide consistent data. Acid was shown to have an interfering effect on the estimation of vasopressin, and the amount of muscle extracted per unit volume of 1M acetic acid used to homogenise the muscle was shown to have a direct effect on the concentrations of vasopressin estimated to be in the sample.

It was concluded that it was not possible to easily assay the extract of foetal muscle in 1M acetic acid and arrive at a reliable estimate of the amount of

vasopressin within a sample. An extraction step to remove acid interference was indicated.

7.1.2 Solid phase extraction of vasopressin from tissues

Octyl-silica (C8 silica) cartridges were used to attempt to identify specific vasopressin immunoreactivity in acid extracts of foetal muscle. The use of C8 silica cartridges was validated by the demonstration that synthetic vasopressin could be reliably extracted from 1M acetic acid with a high recovery, and that extracting vasopressin from extracts of muscle provided a reduced recovery that was judged to be sufficient to justify the use of this method. Positive control samples were extracted in the same way, and levels of vasopressin were similar to published values.

A previously undescribed relationship between the development of the foetus and the amount of vasopressin in the developing adrenal gland was also tentatively observed.

Immunoreactivity apparent in foetal muscle, adrenal glands and pituitary glands was subjected to Sephadex G-25 gel filtration with subsequent radioimmunoassay. Adrenal gland and pituitary gland immunoreactivity was observed to be of a similar mobility to synthetic vasopressin. Immunoreactivity associated with extracts of foetal muscle was observed to elute consistent with it possessing a heavier molecular weight. When large amounts of muscle were extracted and added to the column, small amounts of immunoreactivity were isolated, but these were not sufficient to generate concentrations of vasopressin required to cause fusion of myoblast cells in culture.

It was concluded that the source of vasopressin-like bioactivity which may be responsible for a similar action as that seen in culture could not be isolated from foetal muscle using the C8-silica cartridge method, and therefore the source was unlikely to be vasopressin itself.

7.1.3 Demonstration of vasopressin receptor in foetal skeletal muscle extracts

Vasopressin immunoreactivity was seen to be retained in high molecular weight fractions when extracts of all tissues were subjected to Sephadex G-25 gel filtration. As well as this observation, the proposed model for the involvement of vasopressin (or now, a vasopressin like molecule) required the presence of a vasopressin receptor in foetal muscle cells. Western blotting was carried out using a specific antibody to the human vasopressin V1a subtype receptor in an attempt to demonstrate the presence of such a receptor.

Positive signals could only be identified in older samples. Positive results could not be obtained from other samples from lower gestation fetuses. However, the number of samples examined using this technique was low, and as such can only be regarded as preliminary data.

7.1.4 Immunohistochemical analysis

Immunohistochemistry was used to confirm the observations made in Chapters 3 and 4 of vasopressin immunoreactivity in extracts from human foetal adrenal and adult rat adrenal, and the observations of vasopressin receptor immunoreactivity in extracts of human foetal muscle.

No significant staining was observed in foetal adrenal gland sections. Significant vasopressin staining was seen in the chromaffin cells of the medulla in the adult rat, and this was believed to be the first description of the appearance of vasopressin staining chromaffin cells in the cortex of the adrenal gland.

Results with the vasopressin receptor antibody were inconclusive, even though it was established that the antigens were available in the tissue.

7.2 Discussion

The failure to demonstrate high levels of vasopressin in foetal muscle posed a problem in the investigation of vasopressin as a factor in the differentiation of vasopressin. No experiments described here demonstrated the high concentrations of vasopressin in foetal skeletal muscle described by others (187). As far as was possible, it was attempted to recreate exactly the experimental conditions described by Smith *et al*, since no other reports of similar findings were apparent. Despite this, no evidence of significant amount of vasopressin immunoreactivity, and therefore vasopressin, could be found in any extract of human foetal muscle.

Experimental conditions and the delay before sampling could explain this, and so the experiments do not rule out the possibility that vasopressin genuinely is present in high concentrations in foetal muscle. No data is available on the half life of vasopressin in the foetus, and it could be that it has been broken down in the time before the sample can be frozen.

Taken alone, this should not detract from the theory that vasopressin-like activity is involved in the development of skeletal muscle in the foetus. In this respect, the experiments described have merely shown that the active principle

responsible for the action observed in rat myoblast cell culture, and mimicked by vasopressin, was not immunoreactive to the antibodies used in these experiments. A candidate for this is the vasopressin like peptide described by Hanley, that was isolated from the rat sympathetic nervous system (120). It may be that this substance is the true source of the bioactivity, and further work could be carried out to further characterise the vasopressin like peptide. In particular, the bioactivity of the peptide with reference to the V1a receptor subtype should be established.

Another alternative could be that vasopressin is not required in the concentrations seen *in vitro* to exert this effect *in vivo*. The division of tissues into those that produce vasopressin and use the peptide as an autocrine or paracrine hormone and those that are under the control of circulating vasopressin is still valid. As has been stated, the expression of the V1a receptor seems to be under strict control and can be expressed at different developmental stages. Data defining the *lack* of a receptor at earlier gestation ages were unconvincing. The presence of the receptor in the tissue, therefore, is not a coincidence, and it may be that the designation of vasopressin as a maturation factor in muscle development is correct, but the model of it being expressed by the same tissue is not. The example given earlier of activation in platelet cells is pertinent. Whilst it was initially observed that high doses of AVP were required to activate platelets, Wun and colleagues demonstrated activation of platelets after incubation with physiological concentrations of AVP (95). The overt effects seen in *in vitro* experiments may not reflect the actual required concentration, which could be supplied by circulating levels, and the model proposed for the role of vasopressin in developing skeletal muscle merely requires that its designation as an extrahypothalamic producer of

vasopressin be changed to that of being under the influence of circulating vasopressin.

However, reservations about the veracity of the observations in the rat L6 line exist. It has been pointed out that in normal physiological function, the receptor is not expressed unless it is required as a target for the hormone itself to perform a function. The presence of the hormone is as important a requirement as the expression of the receptor. Whilst the L6 cell line may be a good tool for the study of the physical mechanism of muscle cell fusion, the fact that the cells spontaneously fuse in culture raise questions about the usefulness and accuracy of extrapolating data obtained with these cells to the situation in the whole animal (193). Expression of the vasopressin V1a receptor is a feature of many cancer cells, and it is thought that vasopressin may be a growth factor for certain types of cancer (221). L6 cells are immortalised muscle cells that were treated with a tumour promoter in order to encourage proliferation (192).

Perversely, the very mechanisms that make it conceivable that vasopressin is involved in the process of fusion, are also implicated in the reasoning to suggest that the effects of vasopressin are an artefact. The expression of the vasopressin receptor on the immortalised cells may be a function of the immortalisation process itself. Thus, the worry is that

“...neuropeptides and growth factors use the same intracellular second messengers and that the physiological response of the target cell under certain circumstances is more dependent on the phenotypic expression of the target cell than of the stimuli to which it is exposed” (267).

In addition, experiments where primary satellite cells are used do not seem to justify the case for, or against, vasopressin involvement. Satellite cells are of a

distinct type, and their responses are different to embryonic myoblasts in many ways. Similarity of the response to vasopressin by L6 cells and satellite cells from primary culture may indicate a lack of similarity to embryonic myoblasts.

7.3 Recommendations for further study

The areas of extrahypothalamic expression of AVP and development of skeletal muscle are fascinating, and the exploration of single aspects of either provides views of many more that could be equally productive. The experiments described here represent an initial investigation into some examples of this, and point the way to further research that could be done.

Whilst it may be a point of some contention, the use of foetal human samples to study development of tissues should be continued. Controls are present within the system of recruitment to ensure that the patient is not coerced or pressured into donating or going through with the procedure. For investigators willing to participate in this type of research, it is a valuable source of material.

Experiments carried out to demonstrate and describe the presence of the V1a receptor were confounded by a lack of sensitivity. The first thing to be established is to confirm the tentative observation that vasopressin receptor is only expressed later in gestation. This can be done in a variety of ways. Receptor binding studies are commonly used to characterise receptors, and the isolated membrane could be used to determine the binding seen in foetal muscle. Future research would benefit immensely from harnessing the power of molecular biological techniques. The presence or otherwise of the V1a receptor could quite easily be determined by RT-PCR.

The observation that an increasing vasopressin content was found in foetal adrenal glands is an interesting one. However, this observation was only tentatively stated due to the problems experienced with the sensitivity of the assay used to measure the immunoreactivity, and further work to describe better the content of foetal adrenal glands is necessary. Again, RT-PCR would be a straightforward way to examine expression of the vasopressin gene in the foetal adrenal.

The most successful aspect of this work has been the discovery of the staining of medullary rays for vasopressin in rat adrenal glands. The staining was strongest on the outside of the medulla, and work should be carried out to determine if this was an artefact of the sampling process. In this respect the research would be the same as should be carried out to further elucidate the role vasopressin may play in the development of muscle in the foetus. Similarly, *in situ* hybridisation would supply information in the rat adrenal gland as to the distribution of the vasopressin mRNA in the medulla. If expression is even throughout the medulla, then the theory that vasopressin is distributed where it is most effective would have to be re-evaluated. It would also be interesting to study the distribution of the receptor type in the medulla to see if they have a bias of expression between the inner medulla and the medullary cortical junction.

Finally, a valuable tool in the study of the expression of AVP in the adrenal gland could be the Brattleboro rat. This rat has a base pair deletion in the vasopressin gene and so does not express AVP in the hypothalamus (162). However, AVP immunoreactivity has been demonstrated in the Brattleboro rat testes (268), ovary (122), adrenal gland (161), thymus (149), and in other parts of the brain (163). Different mechanisms of the expression and the quality control processes involved in

translation in these tissues could easily and successfully be studied using these animals.

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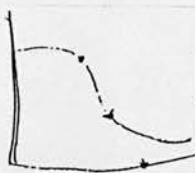
APPENDIX A

APPENDIX A

AVP. ASSAY (T41) 1/10,000 ID 9-6-90

USB
B/T

TC



AVP. ASSAY (T42) 1/20,000 ID 10-6-90

	1/50 Dose	1/10 Dose	1/20 Dose	1/10 Dose		
1) UTERINE MUSCLE	45-225	2610-260	4.26 - 85.2	118.6	1186	= 1119
2) ABOOMINAL "	52-260	22.6-226	3.0 - 60	99.9	999	= 12751
3) MUSCLE END	46-230	17.6-176	4.2 - 84	93.7	937	= 11723
4) HEART MUSCLE	46-34-170	14.9-149	2.18 - 43.6	81.2	812	= 4432
5) MUSCLE	31-155	9.31-93	TOO LOW -	52.3	523	= 1506.1
6) 90/115	41-205	12.5-125	2.05 - 41	84.3	843	= 1101
7) " 139	39-195	15.7-157	1.05 - 21	90.9	909	= 5748
8) " 162	32-160	11.0-110	TOO LOW. 65.2	84.9	849	= 4228
9) " 165	57-114	20.5-205	3.26 - -	105.5	1055	= 11378
10) " 143	27-135	9.6-96	TOO LOW -	67.7	677	= 3766
11) " 082 F	58-290	13.8-138	2.06 - 40.2	99.3	993	= 4876
12) " 078	> CRV	54.7-547	17.5 - 35	340.8	3408	= 20166
13) " 083 F	48-96	11.7-117	TOO LOW -	85.9	859	= 4028
14) " 090 F	60-300	19.9-199	3.04 - 60.8	101.6	1016	= 5214
15) " 092 D	30-100	20.6-206	4.73 - 94.6	123.2	1232	= 12722
16) " 143	68-340	17.1-171	5.04 - 100.8	114.4	1144	= 11027
17) " 155 W	54-270	14.3-143	1.54 - 30.8	94.2	942	= 6379
18) " 130	91-455	16.9-169	3.01 - 60.2	106.7	1067	= 5838
19) " 115	60-300	14.9-149	2.04 - 40.8	99.9	999	= 5202
20) " 120	67-335	20.3-203	4.19 - 83.8	103.17	1032	= 6416
21) " 154	> CRV	66.5-665	19.6 - 392	404.9	4049	= 10322
22) " 123 S	> "	42.8-428	16.8 - 336	322.95	3230	= 5774
23) " 122 S	70-350	22.8-228	5.47 - 109.4	120	1200	= 7767
24) " 138	LOST -	LOST -	LOST -	105.7	-	-
25) " 077	58-290	14.3-143	3.2 - 66	99.8	978	= 5156
26) " 097	85-425	23-230	6.96 - 139.2	121.5	1215	= 1734
27) " 100	51-255	10.7-107	LOW -	84	340	= 1241
28) " 095	71-355	22.5-225	4.64 - 92.8	118.6	1186	= 11772
29) " 139	114-570	23.7-237	7.01 - 140	125.9	1259	= 6002
30) " 156	196-980	24.8-248	7.62 - 152.4	154.3	1543	= 18491

APPENDIX A

Ab T81	1/5 Dia	1/10 Dia	1/20 Dia	Notes	1/10 Dia
31) 90/100	71 - 355	18.3 - 183	3.32 - 66.4		107.8 - (1078)
32) " 097	79 - 395	15.4 - 154	3.19 - 63.8		105.7 - 1057
33) " 112	86 - 430	19.6 - 196	2.85 - 57		99 - 990
34) 81/214	> CRV limit	35.1 - 351	12.3 - 246		142.4 - 1424
35) " 216	64 - 320	18.0 - 180	5.17 - 103.4		113.2 - 1132
36) " 220	84 - 420	23.0 - 230	6.8 - 136		119.3 - 1193
37) " 247 F	81 - 405	20.6 - 206	7.28 - 145.6		126.7 - 1267
38) " 196 S	61 - 305	15.1 - 151	3.33 - 66.6		105.8 - 1058
39) " 243 K	> CRV limit	27.6 - 276	9.4 - 188		130.1 - 1301
40) " 202	91 - 455	17.8 - 178	5.0 - 100		108.6 - 1086
41) 90/34	> CRV limit	24.2 - 242	6.68 - 133.6		112.8 - 1128
42) 89/222	86 - 430	22.0 - 220	6.22 - 124.4		112 - 1120
43) " 224	140 - 700	26.9 - 269	6.27 - 137.4		124.7 - 1247
44) " 055	104 - 520	25.4 - 254	6.23 - 124.6		114.2 - 1142
45) " 065	lost -	lost -	lost -		lost -
46) " 251 F	48 - 240	10.9 - 109	low -		69.3 - 693
47) " 255 K	54 - 270	13.3 - 133	low -		77.4 - 774
48) " 253 W	46 - 230	14.3 - 143	low -		79.4 - 794
49) " 256	59 - 295	14.4 - 144	14.3 - 28.6		80.2 - 802
50) " 261 S	56 - 280	20.4 - 204	2.12 - 42.4		88.9 - 889
51) " 263 F	34 - 170	8.9 - 89	low -		63.9 - 639
52) " 262 F	100 - 500	14.9 - 149	2.05 - 41		100.9 - 1009
53) " 265 F	35 - 175	9.4 - 94	low -		79.3 - 797
54) " 257 S	39 - 195	14.7 - 147	low -		75.7 - 757
55) " 259 F	60 - 300	12.6 - 126	2.44 - 48.8		84.9 - 849
56) " 292 H	45 - 225	11.4 - 114	low -		69.4 - 694
57) " 302 K	56 - 280	19.2 - 192	1.51 - 30.2		79.6 - 796
58) " 297 W	81 - 405	19 - 190	2.70 - 54		91.8 - 918
59) " 303	59 - 295	14.5 - 145	1.69 - 33.8		78.5 - 785
60) " 294 F	78 - 390	21.8 - 218	5.62 - 28.1		116.9 - 1169
61) " 293 F	76 - 380	13.4 - 134	1.42 - 28.4		83.7 - 837
62) " 286 F	89 - 445	22.7 - 227	4.21 - 84.8		107.4 - 1074
63) " 289 J	77 - 385	17.3 - 173	1.48 - 29.6		88.9 - 889
64) " 296 K	low -	low -	low -		low -

APPENDIX A

Ab Tq1	1/5 Dm	1/10 Dm	1/20 Dm	No Tq1	1/10 Dm
65) 89/304	17. - 85	8.9 - 84	Low -		64.7 6.7
66) " 097 S	65 - 300	12.5 - 125	" -		81.6 31.6
67) " 096 H	78 - 390	14.99 - 149.9	" -		79.4 77.4
68) " 307 K	96 - 480	26.4 - 264	3.80 - 76		104.0 102.0
69) 90/016 S	71 - 355	26.9 - 269	3.52 - 70.4		93.2 93.2
70) 89/306 S	72 - 360	28.9 - 289	4.14 - 82.8		92.8 92.8
71) 90/009	47 - 235	16.2 - 162	1.38 - 27.4		32.1 22.1
72) 81/095	118 - 590	17.3 - 173	2.31 - 46.2		93.5 93.5
73) 90/011 F	63 - 315	16.7 - 167	1.99 - 39.8		29.8 39.8
74) 81/311 F		18.6 - 186	3.30 - 76		102.2 102.2
75) 90/012 K	152 - 760	34.5 - 345	6.89 - 137		114.7 114.7

ASSAYED - 9-6-90 (Sun)

Tq1 1 - 1/10,000

125 I AUP - 10-6-90 (Sun)

NRS DARS 13-6-90 (Wed)

10-6-90 (Sun)

Tq2 1/10,000

125 I AUP - 11-6-90 (Mon)

NRS DARS - 14-6-90 (Thurs)

APPENDIX A

MUSENE supernatants.

<u>TUBE NO.</u>	<u>AUT. NO.</u>	<u>AGE</u>	<u>pg/ml</u>	<u>AUT. pg/g tissue</u>	<u>wt</u>	<u>pg/wt</u>	<u>vol.</u>
9	90/105	23/40	22.7	2214.2	0.41	9.35	4
12	" / 072	25/40	630	4134	0.61	2522	4
15	" / 072 D	23/40	230	2127	0.39	830	3.6
* 18	" / 130 S	20/40	123	549.5	0.42	539	4.4
20	" / 120	26/40	246	1273	0.21	1031	4.2
22	" / 123 (S)	24/40	474	4740	0.40	1896	4.0
23	" / 122 (S)	24/40	269	1476	0.22	1210	4.5
29	" / 159	20 "	242	11297	0.09	1017	4.2
30	" / 156	21 "	268	2973	0.30	1070	4.0
34	29/244	12 "	357	10,927	0.13	1428	4.0
* 39	" / 243 (K)	21 "	278	4411	0.27	1192	4.0
* 44	90/055	25 "	1088	1364	0.90	4235	4.2
57	89/302 (K)	19 "	209	2158	0.29	626	7.0
58	" / 247 W	21 "	200	3020	0.26	500	4.5
59	" / 303	27 "	173	921	0.64	559	3.4
62	" / 307 (K)	16 "	289	1000 2723	0.26	723	2.5
69	90/016 S	12 "	281	5229	0.14	732	2.6
70	89/306 S	19 "	321	2967	0.26	771	2.4
* 75	90/012 K	16 "	378	6293	0.15	944	2.5
76	" / 177 (K)	29 "			0.42		4.4
77	29/167 K	16 "			0.12		2.8
78	" / 189 K	16 "			0.05		2.8
82	90/031 J	20 "			0.10		3.0
83	89/107 E	20 "			0.23		4.3
84	" / 160 K	24 "			0.51		3.0
90	" / 121	21 "			0.21		2.0
93	" / 183 K	16 "			0.23		2.0